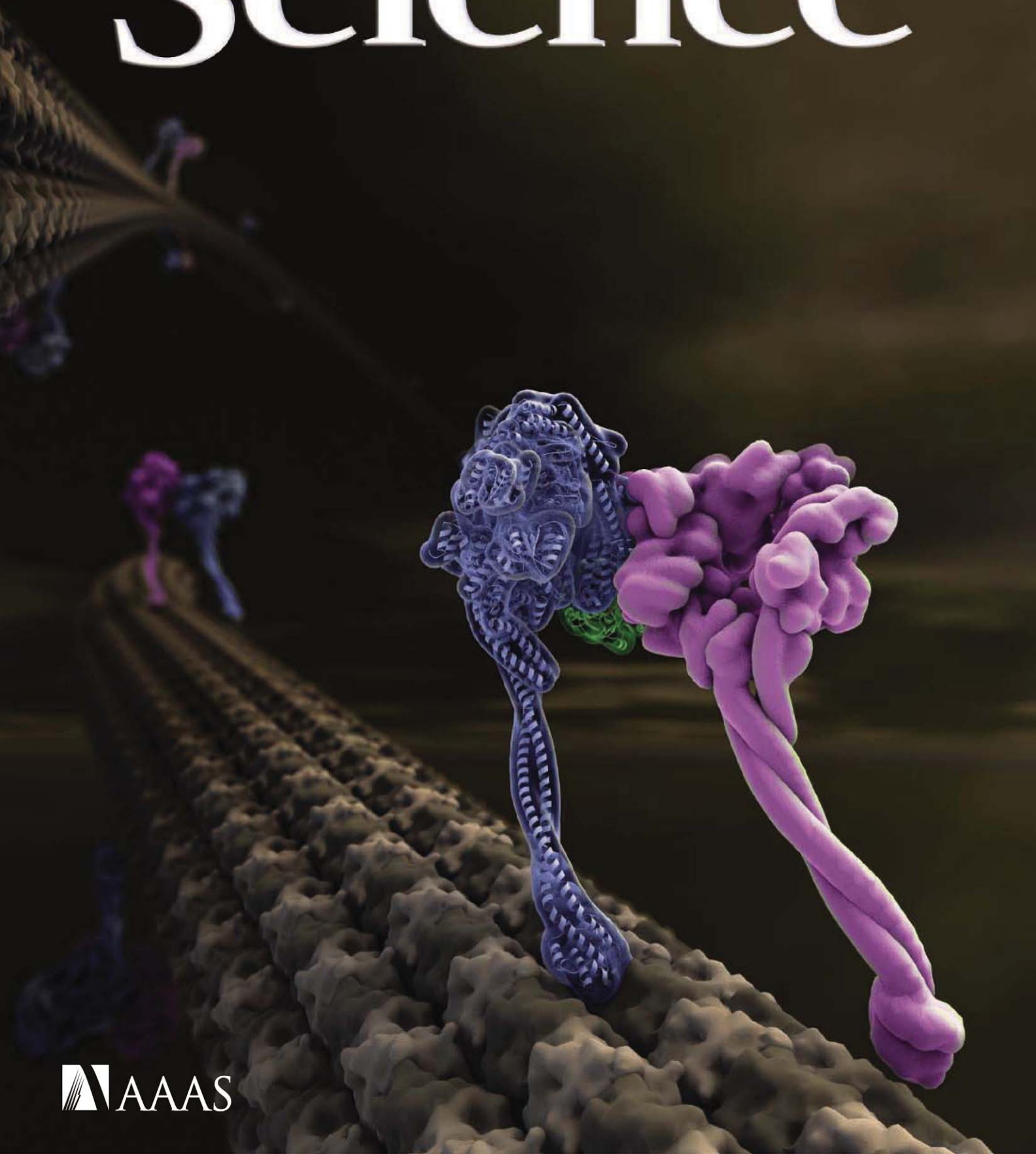


Science

4 March 2011 | \$10



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Call for Symposium Proposals

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We live in a time when collaborations between countries and continents have never been easier, at least from a technical standpoint. A stunning example is the Large Hadron Collider, which is being used by a multinational group of physicists to understand the fundamental building blocks and laws of nature, from subatomic to cosmic. Stores of information and knowledge can be accessed from anywhere by anyone. Remote sensing technology enables the detailed observation of virtually every aspect of our planet's surface, subsurface, and climate. Technology and the Internet are transforming education. Learning is, in principle, available to everyone everywhere.

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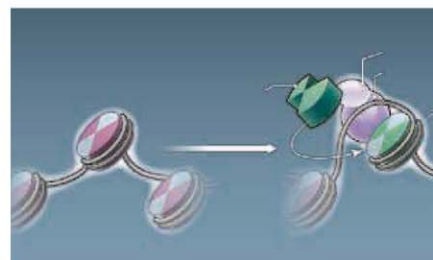
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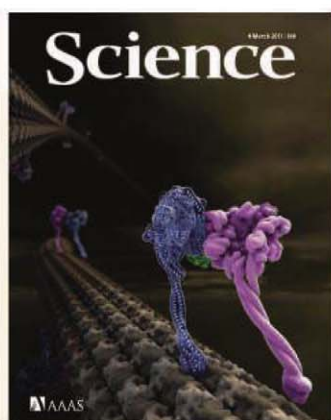
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COVER

Artistic rendering of dynein motor proteins moving along microtubules, based on a crystal structure reported by Carter *et al.* on p. 1159. This study reveals the architecture of the molecular machine that powers the beating of cilia and intracellular transport and provides insight into the mechanism by which energy from adenosine triphosphate hydrolysis is converted into motion.

Image: *Graham Johnson, The Scripps Research Institute and grahamj.com*

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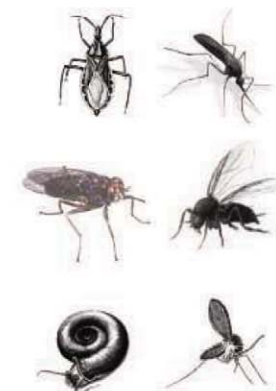
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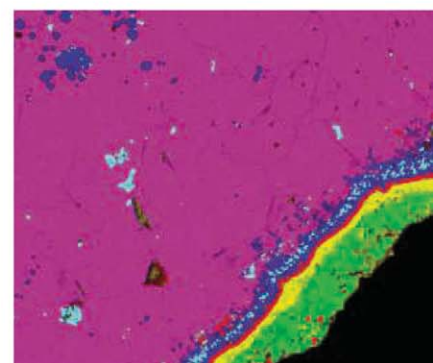
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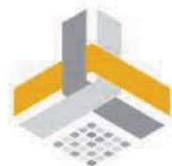
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K. J. Fogle et al.

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10.1126/science.1199702

Selective Inhibition of a Regulatory Subunit of Protein Phosphatase 1 Restores Proteostasis

P. Tsaytler et al.

Guanabenz, a small-molecule inhibitor, protects cells from lethal accrual of misfolded proteins in the endoplasmic reticulum.

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A Virophage at the Origin of Large DNA Transposons

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PAMELA Measurements of Cosmic-Ray Proton and Helium Spectra

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Widespread Persistent Thickening of the East Antarctic Ice Sheet by Freezing from the Base

R. E. Bell et al.

A large fraction of the ice at Dome A, Antarctica, did not form by the usual process of snowfall compaction.

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U. Schenk et al.

Purinergic signaling during inflammation converts immunosuppressive CD4⁺ T cells into proinflammatory ones.

PERSPECTIVE: Astrocytes Shape Axonal Signaling

D. Debanne and S. Rama

Action potential waveform can be altered by astrocyte-mediated activation of neuronal glutamate receptors.

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Find out what APA, Cav-1, and SRK mean in the world of cell signaling.

NETWATCH: The Clickable Guard Cell

Interactive illustrations highlight the signaling pathways that control stomatal opening and closing; in Labs and People.

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Read about current research in network biology and use online tools to explore phosphorylation networks; in Labs and People.

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B. L. Benderly

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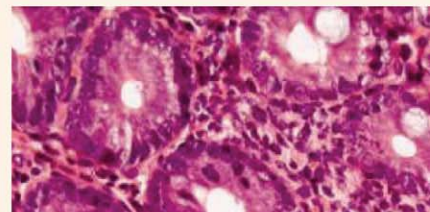
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COMMENTARY: Atherosclerosis Drug Development in Jeopardy—The Need for Predictive Biomarkers of Treatment Response

D. A. Fryburg and M. T. Vassileva

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Controlling immunosuppression in the gut.

RESEARCH ARTICLE: Therapeutic Targeting of SPINK1-Positive Prostate Cancer

B. Ateeq et al.

PERSPECTIVE: A Two-Step Toward Personalized Therapies for Prostate Cancer

A. S. Goldstein et al.

Therapeutic targeting of two proteins that drive tumorigenesis suggests personalized medicine is a possibility for prostate cancer.

RESEARCH ARTICLE: Antisense Oligonucleotides Delivered to the Mouse CNS Ameliorate Symptoms of Severe Spinal Muscular Atrophy

M. A. Passini et al.

Central nervous system-directed antisense therapy ameliorates symptoms in a severe neuromuscular disorder in mice.

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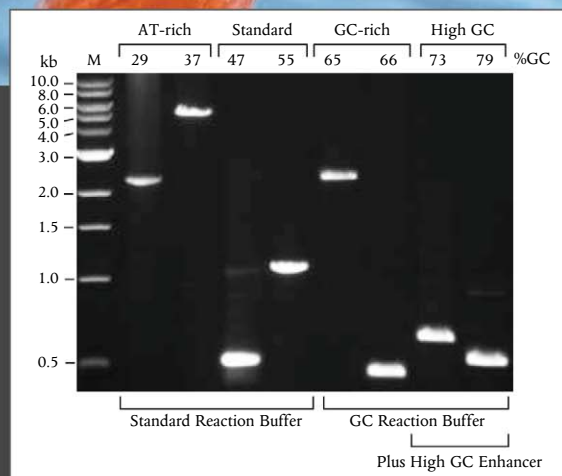
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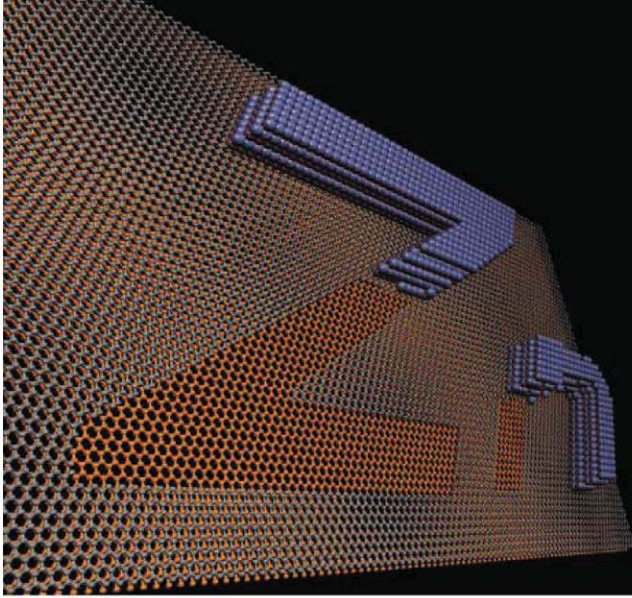
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<< Whittling Away at Graphene Layers

When graphene is used in device applications, it is often supported on a substrate. The support interaction can alter the conductivity of graphene. **Dimiev *et al.*** (p. 1168; see the Perspective by **Gunlycke and Sheehan**) report a chemical route for removing a single graphene layer from various forms of graphene and related materials, such as graphene oxide. They coat graphene with a thin layer of zinc—which can be patterned on the surface—and then dissolve it with dilute hydrochloric acid, which removes the graphene layer.

Netting Pancreatic Cancer Genes

Pancreatic neuroendocrine tumors (PanNETs) are aggressive human cancers that often develop silently and progress to untreatable metastatic disease prior to diagnosis. Using an exome sequencing strategy to identify recurrent somatic mutations in PanNETs, **Jiao *et al.*** (p. 1199, published online 20 January; see the Perspective by **Elsässer *et al.***) find that the most commonly mutated genes, affecting nearly 45% of the tumors, encode proteins implicated in chromatin remodeling. About 15% of the tumors had mutations altering the mammalian target of rapamycin (mTOR) signaling pathway. mTOR inhibitors are already being tested as cancer therapies, so the mutational status of the PanNETs could help to identify which patients are most likely to respond to these drugs.

Join the Queue

Many pathogenic Gram-negative bacteria use type III secretion systems to transfer bacterial effector proteins into eukaryotic cells (see the Perspective by **Stamm and Goldberg**). The core structure is the 3.5-millidalton needle complex (NC) that is organized as rings in the inner and outer bacterial membranes with a needle filament extending out of the cell. Based on cryo-electron microscopy, **Schraidt and Marlovits** (p. 1192) reconstructed a subnanometer structure of the NC from *Salmonella typhimurium* that revealed a 24-fold symmetry for the inner rings and a 15-fold symmetry for the outer rings.

Type III secretion systems work together with a family of translocase proteins, which need to deliver several bacterial effectors to the same target cell in a temporally regulated manner. **Lara-Tejero *et al.*** (p. 1188, published online 3 February) describe a mechanism by which the *Salmonella* type III secretion system sorts its

substrates prior to secretion. A cytoplasmic sorting platform is assembled that is sequentially loaded with the appropriate secreted proteins in a process facilitated by customized chaperones.

Hungry Horses

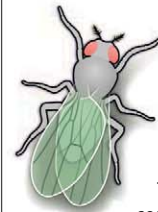
The evolution of horses in the Cenozoic—the last 65 million years—is one of the best-studied prolonged vertebrate radiations. Their evolution is in part thought to be related to changes in available food, and the abrasiveness of that food, as grasslands expanded. **Mihlbachler *et al.*** (p. 1178) used measures of the wear on fossil horse teeth, which have an excellent fossil record, to trace these changes. Most horse populations show relatively low wear, suggesting that dietary pressures were overall low. However, there are several episodes of higher wear in some species, especially during the Miocene, 23 to 5 million years ago, before the appearance of a group of horses with a markedly high crown.

A Visible Sawtooth

Radio frequency (RF) generators can be programmed to emit many differently shaped oscillatory patterns, from sinusoidal waves associated with single frequencies to square and sawtooth shapes that emerge when frequencies spanning multiple octaves are mixed. Analogous manipulation of optical fields is rather more challenging, because laser sources cannot usually produce many mutually coherent components spaced octaves apart. **Chan *et al.*** (p. 1165, published online 20 January; see the Perspective by **Yavuz**) have now succeeded in preparing optical sawtooth and square wave pulses by assembling discrete combs of five harmonics, from the blue to the mid-infrared, derived through Raman shifting in a hydrogen cell and demonstrate a method for characterizing such waveforms based on linear cross-correlation.

Sizing Signals

Growth regulation of the *Drosophila* wing imaginal disc critically depends on the Decapentaplegic (Dpp) morphogen gradient. How a graded Dpp signal is interpreted by cells to control homogeneous tissue growth remains unclear. **Wartlick *et al.*** (p. 1154; see the Perspective by **Le Goff and Lecuit**) address this question by measuring the spatial and temporal



changes of Dpp concentration and signaling activity during the disc growth phase and by quantifying cell proliferation parameters in the discs. Both modeling and experimental

findings suggest that both Dpp concentration and signaling gradients scale with tissue size so that, on average, the onset of mitosis occurs when Dpp signaling levels have increased by 50% since the beginning of the cell cycle.

Tangents to Vector Transmission

A variety of human parasites complete part of their lifecycle in insect vectors and must achieve a balance between prolonging parasite survival and investment in transmission stages. **Matthews** (p. 1149) reviews common themes among a broad group of vector-borne parasites and highlights areas of recent progress, ranging from the different factors that influence a parasite's development in its vector, to the different routes that parasites take as they passage through the insect, to the challenges they face from immune responses and sudden environmental shifts. The combined effect of these processes may result in low rates of insect infection—but not low enough to hinder insect-borne human diseases such as sleeping sickness, malaria, and dengue.

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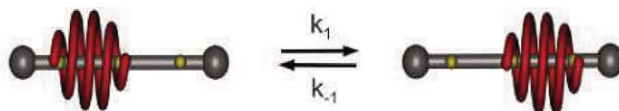
Calcium, aluminum-rich inclusions (CAIs) in meteorites are thought to be the oldest solids in the solar system, preserving clues to events that happened in the early solar nebula, before planets or other solids formed. **Simon *et al.*** (p. 1175) measured oxygen isotope variations within a CAI from the CV3 Allende meteorite. Both the inclusion's core and its rim show systematic variations in relative abundances of oxygen isotopes that approach the range that has been observed for all solids formed in the solar system. The observations imply that, as it formed, this CAI experienced distinct isotopic environments, possibly because it was transported through different regions of the solar nebula.

Dissecting the Centriole

In most animal cells, centrioles template the nine microtubule doublets in cilia and flagella and constitute the core of the centrosome—the microtubule-organizing center. An early stage of centriole assembly is formation of a central hub with nine spokes. **SAS-6** is a protein component of this hub and, based on structural studies, **van Breugel *et al.*** (p. 1196, published online 27 January) show that recombinant SAS-6 can self-assemble into a cartwheel-like structure. Mutagenesis results are consistent with the observed interactions being important in centriole assembly.

Wrap and Slide

In rotaxanes, a central building block in the burgeoning field of nanometer-scale synthetic machines, a molecular ring can shuttle back and forth along a molecular rod but is kept from falling off by bulky stopper groups at each end. **Gan *et al.*** (p. 1172) have designed a variant of this motif in which the ring is replaced by a tightly wound helix. Because the unwinding rate is relatively slow, the helix can slide between binding sites on the rod, despite topologically remaining an open structure. This strategy confers flexibility on the assembly process, which no longer requires kinetically delicate ring-closing and/or stopper-addition reactions.



Improving Old Memories

Can memory be improved? In vivo experiments using pharmacological inhibition of the enzyme protein kinase M ζ (PKM ζ) in the insular cortex have shown that long-term conditioned taste aversion memory can be blocked. Now, **Shema *et al.*** (p. 1207) show that overexpression of PKM ζ in the rat insular cortex enhances memory, including memory that was formed long before the enzyme was overexpressed. The enhancement appears to affect more than a single memory. Thus, modulation of PKM ζ can alter memories, even months after the initial encoding.

Exploiting Ancient Seas

Early colonizers of the New World exploited its wide variety of meat resources—many archeological sites show evidence of hunting of megafauna and smaller animals. Early evidence for widespread marine sustenance has been observed primarily in South America. **Erlandson *et al.*** (p. 1181) now describe sites in the California Northern Channel Islands that show exploitation of marine food for an extended period of time about 12,000 years ago. At that time, these islands were still several kilometers offshore, requiring boat travel to reach them. A variety of tools show evidence of hunting of marine birds, mammals, shellfish, and fish.

Made to Move

Dynein is a large cytoskeletal motor protein, evolved from ring-shaped AAA⁺ adenosine triphosphatases, that moves along microtubules to perform functions such as powering the beating of cilia and transporting intracellular cargo. A long coiled-coil stalk connects the AAA⁺ ring to the microtubule-binding site. **Carter *et al.*** (p. 1159, published online 17 February; see the Perspective by **Spudis**) describe the structure of a dimer of motor domains of yeast cytoplasmic dynein. The structure suggests how conformational changes in the ring associated with adenosine triphosphate binding and hydrolysis might be relayed through the coiled coil to the microtubule-binding domain, leading to a model for the mechanism of motility.

CREDIT: Q. GAN ET AL.

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Research Vital to Economic Growth

IT WAS WITH A MIXTURE OF ASTONISHMENT AND DISMAY THAT I WATCHED AS THE U.S. HOUSE OF Representatives approved H.R. 1, a bill to fund the federal government for the rest of the 2011 fiscal year. Left intact, the massive cuts in research contained in the bill passed on 19 February would effectively end America's legendary status as the leader of the worldwide scientific community, putting the United States at a distinct disadvantage when competing with other nations in the global marketplace. Other countries, such as China and India, are increasing their funding of scientific research because they understand its critical role in spurring technological advances and other innovations. If the United States is to compete in the global economy, it too must continue to invest in research programs.

As the Under Secretary for Science at the Department of Energy (DOE) in the administration of George W. Bush, I can personally attest that funding for scientific research is not a partisan issue—or at least it shouldn't be. The cuts proposed in H.R. 1 would reverse a bipartisan commitment to double the science research budgets of the National Science Foundation, the DOE Office of Science, and the National Institute for Science and Technology over 10 years. These are national goals supported by both Presidents Bush and Obama, and they were affirmed as recently as last December in the America COMPETES Act.

The spending cuts included in the bill would have a devastating effect on an array of critical scientific research. For example, H.R. 1 removes \$900 million from the budget for the Office of Science, the basic research arm of the DOE—a reduction of some 20%. The bill specifically targets the Office of Biology and Environmental Research, slicing its budget by 50%; reductions that would all but eliminate funding for the office's three Biological Research Centers, the hope for developing transportation fuels derived from plant cellulose. The hugely successful Energy Frontier Research Centers, which support activities based at 28 universities and 16 national laboratories, would be cut in midstream. The university centers support 1300 students working on the conversion of sunlight and heat into electricity, improved efficiency of photosynthesis in plants for the production of fuels, and enhanced combustion efficiency to increase mileage for automobiles. The work now at risk at the national laboratories includes projects to improve solid-state lighting and the conversion of coal into chemicals and fuels. This research is vitally important if the United States is to be a leader in transforming how humans get and use energy globally, in a way that maintains societal and economic viability.

To make matters worse, the bill would also destabilize the large-scale scientific facilities operated by the DOE's Office of Science. These research projects include the country's work with powerful light sources (which other countries are copying en masse), so vitally important to the U.S. biological, medical, and materials communities. Also included are the nation's remaining accelerators, responsible for advances in the high-energy and nuclear science communities; its spallation neutron source and nanotechnology centers, critically important to both university and industrial communities; and the quest for environmentally benign unlimited energy through investment in the International Thermonuclear Experimental Reactor.

The budget deficit is serious. But escaping from its clutches requires economic growth as well as budget reductions. Well over half of U.S. economic growth in the past century can be traced to investments in science and technology. To compete in the global economy, the United States must remain a leader in science and technology. For that to happen, the Senate must restore funding for science in the fiscal year 2011 budget. Failure to do so would relegate the United States to second-class status in the scientific community and threaten economic growth and prosperity for future generations of Americans.

— Raymond L. Orbach



10.1126/science.1204760

CHEMISTRY

Calcium Catalysis

Synthetic asymmetric catalysts have traditionally comprised transition metals bearing chiral ligands, though over the past decade, free amines, phosphate diesters, and urea derivatives have emerged as increasingly effective alternatives. The phosphate class of organocatalysts tends to rely on ion pair formation between a protonated substrate and a residual phosphate anion bearing a chiral, often polycyclic aromatic carbon framework. Zheng *et al.* were exploring the application of this type of catalyst toward asymmetric chlorination of phenyloxindoles, and they found that a particular phosphate diester afforded high enantioselectivity just after purification by silica gel chromatography, yet minimal selectivity after an acid wash. This result led them to screen phosphate salts with a variety of cations, culminating in the discovery that calcium—a biochemically critical ion that has found comparatively few applications in synthetic catalysis—proved the optimal counterion for conferring selectivity. Though the mechanism remains unclear, the authors posit possible activation of the chlorine source (*N*-chlorosuccinimide) by calcium coordination, concomitant with oxindole activation by the phosphate. Phenyloxindoles with a range of substitution patterns were chlorinated in nearly quantitative yields within half an hour at room temperature, with enantioselectivities generally exceeding 90%. — JSY

J. Am. Chem. Soc. **133**, 10.1021/ja109824x (2011).

CLIMATE SCIENCE

Reservoir Doubts

As the last ice age came to an end, the concentration of atmospheric CO₂ increased rapidly, and its ¹⁴C content declined precipitously between 17.5 and 14.5 thousand years ago. The favored explanation for this drop in the proportion of ¹⁴CO₂ is that a large amount of old carbon—that is, carbon whose inventory of radioactive ¹⁴C had been depleted with age—was outgassed from a deep ocean reservoir where it had been isolated for many thousands of years. That scenario makes good intuitive sense, but conclusive evidence for a reservoir of aged carbon has not yet been found. Hain *et al.* use an 18-box ocean model to simulate the history of deglacial CO₂ and evaluate the likelihood that an old deep-ocean CO₂ reservoir was in fact the source of the observed ¹⁴C anomaly. They conclude that such a transfer of carbon was unlikely, based on inconsistencies



Although a variety of factors contribute to global wildfire activity, climate is thought to be a major determinant. Ohlson *et al.* now find that tree species composition has had at least as strong an influence as climate on wildfire activity over time in late Holocene boreal forests. By analyzing humus and peat records in northern Europe, the authors show that the invasion of Norway spruce, *Picea abies*, southwesterly across the region starting 4500 years ago was accompanied by a reduction in the frequency and severity of fire. The presence of Norway spruce would tend to make the forest more dense and shaded in the drier summer months, which would increase the local humidity of the understory and inhibit the spread of fire. Although the spruce invasion corresponded with a period of cooler and wetter climate, the tight spatial correlation of the arrival of spruce and the reduction in fire in specific locations argues for a key role of species composition in governing the fire regime. In turn, the reduction in fire would have led to other ecological consequences, notably increased sequestration of carbon by the vegetation and the establishment of other species requiring longer-term continuity. Thus, the results show how changes to the dominant forest species can exert a cascading effect on the regional ecology for centuries or millennia to come. — AMS

J. Ecol. **99**, 395 (2011).

with other records such as those of the ¹⁴C history of other depths and regions of the ocean, ocean carbonate and oxygen changes over the interval, and constraints on mixing imposed by ocean circulation. — HJS

Geophys. Res. Lett. **38**, L04604 (2011).

BIOCHEMISTRY

Minding Their Ps and Ns

In the mid-20th century, Alfred Redfield posited that the bulk ratio of nitrogen to phosphorus atoms (N:P) in marine microorganisms should maintain a relatively constant value of ~16. Work since then has shown that the

ratio indeed remains relatively constant across many environments and time scales, including deep oceans and coastal waters, but questions remain about whether innate biochemical or environmental factors are responsible. Loladze and Elser compiled literature values of nutrient ratios in prokaryotic and eukaryotic microorganisms, which, combined with a theoretical model, suggest that the N:P ratio is determined by a balance of maximum macromolecule biosynthesis rates—specifically for nitrogen-rich proteins and phosphorus-rich ribosomal RNA. Although the analysis considered cases in which growth rates were optimal, an N:P ratio of ~16 isn't necessarily always desirable for efficient

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growth; communities in environments where the paucity of nitrogen or phosphorus limits growth may have optimal N:P ratios that are shifted away from 16. Because one of these two nutrients is often the main limiting growth factor in aquatic and terrestrial systems, observable deviations in N:P ratios would therefore provide insight into the biogeochemical processes that shape microbial community structure. — NW

Ecol. Lett. **14**, 10.1111/j.1461-0248.2010.01577.x (2011).

BIOMEDICINE

Keep Fit

Recent work has implicated mutations in mitochondrial DNA (mtDNA) in aging, and in humans, endurance training has been linked to health benefits, including increased life expectancy. Safdar *et al.* wanted to see if these processes could be linked in a strain of mice prone to mtDNA damage and who also exhibit a reduced life span. In these mice, a regime of endurance training—running for 45 min three times a week over 5 months—induced mitochondrial biogenesis, increased mitochondrial respiratory capacity, and prevented mtDNA damage. Furthermore, the trained mice no longer exhibited premature mortality or other symptoms associated with accelerated aging including fat loss, muscle loss, anemia, and



graying fur (compare 30-week-old endurance-trained mouse, left, to 30-week-old control mouse, right). Future work will be required to see if similar regimes can protect and “rejuvenate” human mitochondria and mitigate the effects of normal or pathological forms of premature aging. — SMH

Proc. Natl. Acad. Sci. U.S.A. **108**, 10.1073/pnas.1019581108 (2011).

BIOCHEMISTRY

Rethinking Riboswitch Regulation

The enzyme glucosamine-6-phosphate (GlcN6P) synthase (GlmS) is an important point of metabolic control in the synthesis of amino sugars: essential precursors in the biosynthesis of bacterial cell walls. GlmS gene expression is

regulated by the *glmS* riboswitch, a sequence in the 5′ untranslated region of the mRNA that is also a ribozyme. Binding of GlcN6P activates *glmS* self-cleavage and this negatively regulates expression of GlmS.

Watson and Fedor now reveal another level of complexity to riboswitch regulation. By growing yeast on various carbon sources and then measuring the ligand concentration dependence of intracellular cleavage kinetics, they found that products of hexose metabolism can competitively inhibit GlcN6P-induced self-cleavage. These results were confirmed in the Gram-positive bacterium *Bacillus subtilis*, the natural biological context for the riboswitch. These findings suggest that the *glmS* riboswitch might integrate chemical signals to modulate gene expression in response to the overall metabolic state of the cell. — VV

Nat. Struct. Mol. Biol. **18**, 10.1038/nsmb.1989 (2011).

MOLECULAR BIOLOGY

Export Examined

Many RNAs made in the cell nucleus function in the cytoplasm and so must be exported from the nucleus. Pre-microRNAs (pre-miRNAs) are exported from the nucleus via the karyopherin exportin-5 and are processed to their mature (miRNA) form in the cytoplasm by the Dicer protein, where they play a critical role in regulating gene expression. Bennasser *et al.* find that export of the Dicer mRNA from the nucleus of human cells also requires exportin-5 protein. The expression of exportin-5 is limiting, and overexpression of a substrate miRNA is able to saturate the exportin-5 export pathway. This leads to a decreased association between exportin-5 and its other substrates—such as the Dicer mRNA, which results in reduced amounts of Dicer protein in the cell. Thus, there is cross-regulation between pre-miRNAs and their processing enzyme, Dicer, which could help balance the amounts of the enzyme and its substrate.

Upon infection, adenovirus produces large quantities of two small structured RNAs, which are exported by exportin-5 and can saturate the export pathway and reduce Dicer expression. Knockdown of either exportin-5 or Dicer enhances the growth of a replication-defective adenovirus, hinting that pathogens might also be able to exploit the regulation of Dicer mRNA levels by exportin-5 and, as a result, miRNA and siRNA levels. — GR

Nat. Struct. Mol. Biol. **18**, 10.1038/nsmb.1987 (2011).



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Science Careers

From the journal *Science*



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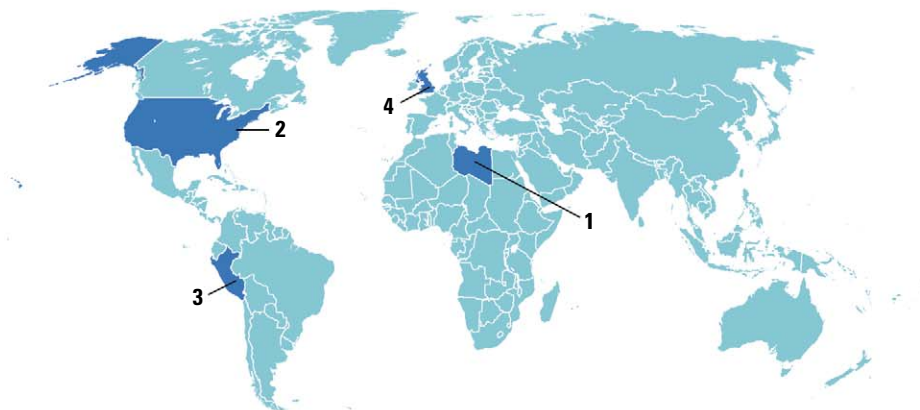
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AROUND THE WORLD



Libya 1

Exodus of Fossil Hunters

The sudden outbreak of violence in Libya has caught some foreign researchers by surprise. American paleoanthropologist Noel Boaz was among a half-dozen visiting academics hunkering down for several days in a house owned by the Libyan International Medical University in Benghazi, where they were teaching. Although they escaped over the Egyptian border at midnight on 23 February, Boaz (now back in the United States) worries about the fate of important animal fossils discovered during years of field work in Sahabi and currently stored in a fort in Tripoli. Archaeologist Barbara Barich of Sapienza University of Rome says Italian colleagues beat a similarly hasty retreat aboard an Italian military aircraft.

Meanwhile, a French-American team searching for early anthropoids has abandoned plans to return to the field site of Dur At-Talah (pictured) in March, says paleontologist K. Christopher Beard of the Carnegie Museum of Natural History in Pittsburgh, Pennsylvania. And things are looking uncertain for dinosaur paleontologist Paul Sereno of the University of Chicago, who just signed an agreement in January for “large-scale exploration and research” across Libya. Sereno, who wasn’t planning to go to the field until November, says he still hopes to work there in 2012.

Washington, D.C. 2

Vaccine Maker Wins In High Court

The U.S. Supreme Court came down firmly on the side of a vaccine manufacturer last week, ruling 6-2 with one abstention that the parents of a teenager who was apparently seriously injured by a vaccine she was given as a baby aren’t entitled to sue the maker, Wyeth.

The parents of Hannah Bruesewitz argued that she had received a “scientifically outmoded” DTP (diphtheria, tetanus, pertussis) vaccine that Wyeth had decided not to update. But Antonin Scalia and five other justices concluded that the National Childhood Vaccine Injury Act of 1986, which sought to protect manufacturers from excessive liability claims while preserving a family’s right to be compensated for vaccine injuries, did not intend for companies to be liable for such so-called design defects.

In a dissenting opinion, justices Sonia Sotomayor and Ruth Bader Ginsburg accused the court of imposing “its own bare policy preferences” and said it was important for companies to be pushed, at times by litigation, to improve on existing vaccines. “Nothing in the text, structure, or legislative history remotely suggests that Congress intended” to prevent this, they wrote.

<http://scim.ag/vac-ruling>

Madre de Dios, Peru 3

Government Cracks Down On Destructive Mining

Peru is finally getting tough with the illegal gold-mining operations that are destroying thousands of hectares of rainforest near the headwaters of the Amazon River. To avert further environmental devastation, the gov-

ernment on 20 February dispatched members of its armed forces on a 6-week mission to Madre de Dios in southeastern Peru to eradicate illegal dredgers—heavy machines that scoop up sediments from river bottoms. As *Science* went to press, the government had so far burned and destroyed 19 dredgers.

Scientists have dubbed Madre de Dios one of the world’s biodiversity hotspots. It harbors important fish spawning grounds and the second-richest butterfly community ever documented in the world. But soaring gold prices have lured an estimated 64,000 migrants from other parts of Peru to work as individual miners or for larger operations, scouring riverbeds, stripping away rainforest and soil, and extracting gold from sediments with some 32 tons of toxic mercury annually. Today, 150,000 hectares of the region are virtual dead zones. “It looks like Mordor,” says Alex Chepstow-Lusty, a pollen scientist at the French Institute of Andean Studies in Lima.

Milton Keynes, United Kingdom 4

Computing Pioneer’s Papers To Stay in Public Collection

Rare offprints of the scientific papers of mathematician and World War II British codebreaker Alan Turing have, with the help of generous donations, been bought for the Bletchley Park Trust, the museum housed in the wartime codebreaking center where Turing helped crack the German Enigma code.

Little remains of Turing’s personal belongings and papers, so these offprints—given by Turing to friend and fellow codebreaker Max Newman and with notes in Turing’s hand—were expected to fetch at least £300,000 at auction. But after donations from the U.K.’s National Heritage Memorial Fund, Google, and the public, Bletchley Park was able to buy them.

Turing is considered one of the founders of modern computing, and at Bletchley he developed the initial design of the Bombe computer used to decrypt Enigma-encoded messages. The offprints, along with Newman’s guest book containing the signatures of Turing and other Bletchley veterans, “represent a piece of our code-breaking heritage,” says Kelsey Griffin, director of the Bletchley museum.



NEWSMAKERS

Three Q's

Nathan Myhrvold has been a postdoc for physicist Stephen Hawking and chief technology officer at Microsoft, but these days he's turning heads with his innovations in the kitchen. On 14 March, he and two chefs will publish *Modernist Cuisine: The Art and Science of Cooking*, a six-volume, 2438-page opus on a new style of cooking that uses high-tech gadgetry and ingredients to concoct dishes as unlikely as geoduck noodles and deep-fried mayonnaise.

**Q: What's your favorite kitchen gadget?**

We love rotor-stator homogenizers. It's [a] piece of laboratory equipment that's fantastic for making very fine purees or very fine-grained emulsions.

Q: Is cooking becoming more of a science these days?

Inquisitive chefs have always been scientists ... [and] there's a huge section of the book explaining how traditional cooking methods actually work. ... Here's one thing that really surprises people. A lot of recipes have you plunge stuff into ice water to stop the cooking. ... It's based on a mental fallacy that's very similar in spirit to the idea that Galileo

disproved, that heavier objects fall faster. In fact, the peak temperatures will be almost exactly the same ... if you put the food out on the counter versus plunging it in ice water.

Q: If you had to compare the book to a scientific work, what would it be?

Gravitation by [Charles] Misner, [Kip] Thorne, and [John] Wheeler ... was a huge inspiration to me with the cookbook. It was big and comprehensive and had historical sidebars. ... I love that. ... As an example, we have a sidebar on Joseph Fourier and the laws of heat conduction. And we have a little biography of James Watt, because everyone knows their appliances are rated in watts, so we explain what that means.

Musical Chairs at DOE

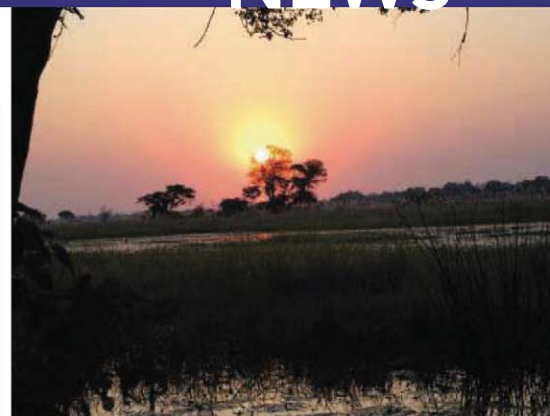
The top echelon at the Department of Energy seems to be in perpetual motion. Last week, Secretary Steven Chu gave **Arun Majumdar**, an engineer who heads the Advanced Research Projects Agency-Energy, a second job: acting under secretary for energy. Majumdar will take over from departing **Cathy Zoi** until a permanent replacement is found. Zoi, like Majumdar, was filling in temporarily while pulling double-duty—she also ran the Office of Energy Efficiency and Renewable Energy. Her deputy, **Henry Kelly**, former head of the Federation of American Scientists, will get that job.

<http://scim.ag/doe-changes>

Ötzi's New Look

Nearly 20 years have passed since the 5300-year-old body of Ötzi the Iceman was discovered in a melting alpine glacier by two hikers just on the Italian side of the Austria-Italy border. In celebration, the South Tyrol Museum of Archaeology in Bolzano, Italy, where the Iceman's mortal remains are on display, opened a massive Ötzi exhibit this week featuring a brand-new reconstruction by the Dutch master paleoartists Adrie and Alfons Kennis (*Science*, 10 July 2009, p. 136).

The bearded Iceman appears particularly fit and trim, as he might have looked before an enemy arrow apparently brought him down. On 18 September, the night before the discovery's 20th anniversary, the museum will host a late-night birthday bash for Ötzi. But if you miss the party, you can still see the exhibit until 15 January 2012.



FINDINGS

Visions From Africa

On an African savanna 10 million years ago, our ancestors may have gazed upon a view much like this one. A new study suggests that this complex scenery influenced the mosaic in which the human retina's red, green, and blue light-sensitive cone cells are arranged.

Studies in other species have suggested that cone cells evolve into a configuration that most efficiently takes in an animal's environment. To find out whether the same happened in human eyes, physicist Gasper Tkačik, neurobiologist Vijay Balasubramanian, and colleagues at the University of Pennsylvania created a database of more than 5000 high-resolution photographs, including this one, taken at various locations in a primate habitat in Botswana, the general region where humans likely evolved. The researchers measured the color information contained in these images and created an algorithm to predict which pattern of cone cells would extract the most information. That turns out to be the one found today in our retinas, which have an overabundance of red and green cones. The study and the image database, described in a paper on the preprint arXiv, may be useful for researchers trying to create machines with humanlike vision. <http://scim.ag/eyes-evolve>

Stage Set for Sixth Mass Extinction of Animals

By consulting the fossil record to see how quickly different creatures have gone extinct in the past, a team of scientists has found that animals are going extinct so rapidly that we are on the brink of a mass extinction. Mass extinctions—in which more than three-quarters of the species on Earth disappear—have taken place five times in the past 540 million years. But if all of the animals that are currently endangered, critically endangered, or vulnerable actually >>

BY THE NUMBERS

21% Percentage by which India is boosting science and technology spending in its new budget, announced this week.

75% Percentage of the world's coral reefs threatened by global warming, overfishing, and other factors, according to the World Resources Institute.

30 per week The rate at which results of clinical trials are being submitted to ClinicalTrials.gov. If a federal mandate were followed, the rate would be 100 records per week, according to a study in *The New England Journal of Medicine*.

go extinct, their loss will add up to a sixth mass extinction, according to a report published this week in *Nature* by paleobiologist Anthony Barnosky and his colleagues at the University of California, Berkeley. The researchers estimate that this mass extinction could arrive as soon as 300 years to 2200 years. But Barnosky says there's still time to fend it off by protecting these endangered animals from invasive species, habitat loss, disease, and global warming.

<http://scim.ag/mass-extinction>

Weird Matter Seen In Neutron Stars

Astrophysicists have long speculated that nuclear matter inside neutron stars might flow without resistance—much as electricity does in superconductors. Now, two teams say they have direct evidence of such bizarre “superfluidity” in a neutron star.

When a superconductor is cooled nearly to absolute zero, the electrons in it form hard-to-break “Cooper pairs” that flow without resistance. In theory, inside a neutron star—the superdense core of a massive star that exploded as a supernova—neutrons should pair in a similar way.

Astronomers have seen indirect hints

Random Sample

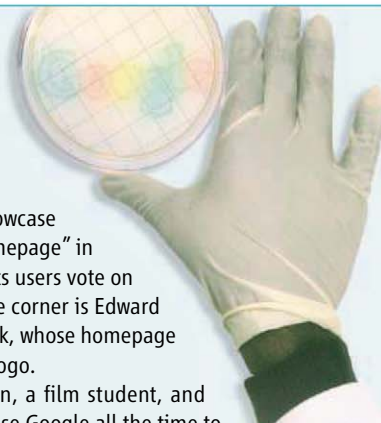
Geek coli

Can two microbiologists win a cage match against tennis star Maria Sharapova? You can help decide.

Google Demo Slam, a contest for videos that showcase Google features such as “translate” and “custom homepage” in creative ways, pits one video against another and lets users vote on a champ (<http://www.demoslam.com/>). In the science corner is Edward Johnson of Eastern Virginia Medical School in Norfolk, whose homepage entrant is an *Escherichia coli* rendition of Google's logo.

Johnson learned about the contest from his son, a film student, and decided to enter on a whim. After all, he says, “We use Google all the time to find out scientific things.” He and his graduate student Clayton Wright dyed lab bacteria different colors, streaked them onto an agar plate in the shape of the Google logo, and let them grow.

Google loved the result, which users can now set as their homepage background. But even if voters deem one of the other entries, such as Sharapova's Russian language voice search, more infectiously charming, Johnson relished the experience. “It's great to see the comments on YouTube,” he says. “There are a lot of people out there who don't know much about science but have a genuine interest.” He says fans of the viral video have even contacted him personally to hear about his HIV and cancer research.



of such neutron superfluidity. Now, a team led by Dany Page, an astrophysicist at the National Autonomous University of Mexico in Mexico City, say NASA's orbiting Chandra X-ray Observatory has bolstered the case. Chandra's observations of the young neutron star Cassiopeia A show that its temperature of about 2 million kelvin plummeted 4% in 10 years. That huge cooling rate—the first ever measured in real time for a neutron star—shows that Cooper pairs are forming, the researchers reported online last week in *Physical Review Letters*. That's because when neutrons pair, they release energy that can increase the emission of neutrinos, the star's main cooling mechanism. A team led by Dmitry Yakolev of the Ioffe Physical-Technical Institute in St. Petersburg, Russia, has a similar paper in press at the *Monthly Notices of the Royal Astronomical Society*. <http://scim.ag/neutron-star>

Indonesia's Mud Volcano To Flow On and On

Since it roared to life in May 2006, a mud volcano near Indonesia's coastal city of Sidoarjo has swallowed homes, rice paddies, factories, and roads, killing 15 people, displacing 40,000, and harming the livelihoods of many more. Will it ever stop? Not anytime soon. A new study online in the *Journal of the Geological Society* predicts the volcano, nicknamed Lusi, will continue



spewing significant amounts of mud for at least another 2 decades.

Richard Davies, a geologist at Durham University in the United Kingdom, believes that the eruption is driven by pressurized water from a deep aquifer beneath an impermeable rock layer. He argues that a well bore pierced the impermeable rock, allowing water to gush up and sweep overlying mud to the surface. Based on the volume of mud ejected after 1 year and 3 years, and assumptions about the size of the aquifer, Davies and colleagues estimate that mud will be flowing for 26 years and that the ground around Lusi will subside up to 475 meters. And that may be an underestimate—other researchers contend the flow could last even longer. <http://scim.ag/lusi-mud>

ASTROPHYSICS

Scientists Fear WFIRST Will Be Trailing the Pack

The 1990s were a decade of glory for U.S. astronomers, including the discovery of dark energy and planets outside the solar system. But the instrument that they say would keep them at the forefront—even its name reflects that aspiration—may be too costly for the U.S. government to build.

Last August, a National Academies panel charged with ranking the priorities of U.S. astronomers for the next decade identified the Wide-Field Infrared Survey Telescope (WFIRST) as its top choice in the category of space observatories. The panel recommended that NASA launch the \$1.6 billion mission by 2020 as the best way to advance the study of dark energy and the search for new extrasolar planets.

But hopes of realizing that goal appear to be fading. The ballooning cost of the James Webb Space Telescope (JWST), and the resulting delays in its launch, have all but guaranteed that the space agency will not be able to deliver WFIRST by 2025, much less the end of the decade. And NASA has rejected a backup plan that would have given it a 20% stake in a similar European dark energy mission. That decision, announced last week, leaves U.S. astronomers with the prospect of being marginalized in the next decade.

The president's 2012 budget for NASA includes \$4 million for conceptual development of WFIRST. But officials say that any commitment to WFIRST will have to wait until NASA completes an internal review this summer of JWST, whose \$6.5 billion price tag and 2017 launch date are, respectively, \$1.5 billion higher and 3 years later than previously scheduled.

If NASA can't fund WFIRST in this decade, says astrophysicist Roger Blandford of Stanford University in Palo Alto, California, who chaired the recent decadal study by the National Academies' National Research Council, "then I will be bitterly disappointed on behalf of the whole community, which put so much into making tough choices to create what ought to be an executable program. It would reflect very badly on future U.S. scientific leadership and demoralize the outstanding young people" working on dark energy and exoplanet research, he adds.

What Blandford and others fear is that WFIRST will end up W-second to a European dark energy mission called Euclid. The \$1 billion mission, to be launched in 2018, is one of three proposals before the European Space Agency (ESA), which is expected to pick two this fall to go forward.

Although the two telescopes will both pursue dark energy, they are quite different from each other. WFIRST is a 1.5-meter infrared telescope that will tap all three cosmological probes for investigating dark energy—gravitational weak lensing, baryonic acoustic oscillations (BAO), and type I supernovae—in addition to using microlensing to find exoplanets. Euclid is a 1.2-meter telescope that will focus on weak lensing and BAO.

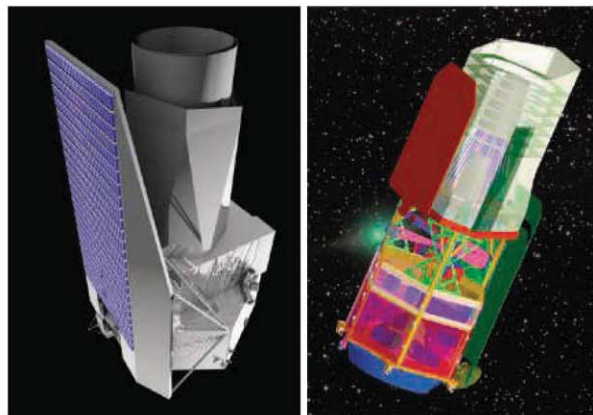
Until last summer, NASA officials planned to enter into a junior partnership with ESA on the Euclid project in addition to going ahead with WFIRST. That arrangement seemed to guarantee U.S. participation in whichever dark energy survey was launched first. But the decadal survey panel recommended that the United States invest in only a U.S.-led dark energy mission, whether that be WFIRST or a partnership with Europe that preserved WFIRST's "science program" and allowed the United States to take the leading role.

After last fall's update on Webb, the decadal survey panel reviewed the options for a dark energy mission in light of Webb's troubles. In January, the panel concluded that a 20% partnership with ESA on Euclid was not advisable. The panel argued that it would prefer to see NASA invest in other projects from the decadal survey if it couldn't fund WFIRST within the decade or play a leading role in any partnership.

NASA has already acted on that advice, says the agency's head of astrophysics, Jon Morse. "We have informed ESA that we will not be pursuing a 20% option [in Euclid] at this time," Morse told the interagency Astronomy and Astrophysics Advisory Committee at

its meeting last week. Instead, he said, NASA would ask ESA to consider a combined mission merging WFIRST and Euclid.

But ESA seems unlikely to accept such an offer at this time. Speaking to a panel of the NASA Advisory Council last September, Fabio Favata, ESA's coordinator for astronomy and fundamental physics missions, said a lot of work had already been done on Euclid and that merging the missions would require going back to the drawing board. "I think the U.S. lost a real opportunity in passing up a 20% partnership with Euclid," says Jason Rhodes, an astrophysicist at the Jet Propulsion Laboratory in Pasadena, California, and a member of the Euclid Consortium helping to plan the mission. "It was a big enough stake that the U.S. could have influenced the design and execution of Euclid."



Dueling telescopes. ESA's Euclid mission (left) could get the green light while NASA delays WFIRST.

The community's opposition was driven by concerns that "this would be the beginning of a slippery slope where the U.S. would end up ceding leadership to the Europeans in astrophysics, just as leadership was ceded in particle physics," explains James Green, an astrophysicist at the University of Colorado, Boulder, and co-chair of WFIRST's Science Definition Team. "The question is, Do we [the United States] risk getting nothing at all?"

However, Green believes that a merger is not out of the question. If Euclid were not selected for funding by ESA in October, he and others speculate that the European architects of the proposal might welcome a 50% partnership with NASA.

Morse says it is too early to "draw the curtain" on WFIRST or on a merger. NASA's new plan for completing JWST and ESA's decision on whether to fund Euclid will "show us the path to the future," he says.

—YUDHIJIT BHATTACHARJEE

ARCHAEOLOGY

Do Island Sites Suggest a Coastal Route to the Americas?

About 12,000 years ago, on beautiful Santa Rosa Island off the coast of California, a group of prehistoric hunters bagged geese, cormorants, and albatross, perhaps using crescent-shaped stone points to stun the birds. They also caught a rainbow array of fish, including surfperch and rockfish, probably spearing them with barbed stone points also used to hunt seals. These sea goers must have crossed 10 kilometers of open water from the coast near what is now Santa Barbara to reach the island, and they also visited nearby San Miguel Island, where they raked in bountiful amounts of red abalone, mussels, snails, and crabs.



tral riddle in archaeology: How did prehistoric peoples, once they arrived in Alaska via a land bridge from Asia, journey south to the Americas? In recent years, some researchers have suggested that they came by boat down the coast of North America rather than through the continent's interior. But evidence for this hypothesis has been thin.

"The new finds are remarkable," says archaeologist Charlotte Beck of Hamilton College in Clinton, New York. "They add important support to the coastal route argument." But other researchers argue that the new sites are too young to tell us much about the routes that the first Americans took more than 14,000 years ago. It's possible that the Channel Islands people originally stemmed from inland populations that later moved to the coast, these researchers say. "The most parsimonious interpretation is that these were seasonal forays by mobile Paleoindian groups" living on the mainland, says archaeologist David Yesner of the University of Alaska, Anchorage. "It is difficult to link them to a coastal migration."



The maritime life. Prehistoric peoples living on the islands of San Miguel (foreground) and Santa Rosa (far distance) used stone points (inset) to hunt birds, fish, and seals.

On page 1181 of this issue, archaeologist Jon Erlandson of the University of Oregon, Eugene, and his colleagues detail the remains of these ancient hunts: thousands of bird, fish, and sea-mammal bones, as well as shells and the characteristic stone points.

Some say that this evidence of early American coastal life offers clues to a cen-

On most of the North American mainland, the earliest signs of Paleoindians are so-called Clovis points, elegant fluted arrow or spear points dated no earlier than 13,000 years ago. Researchers once assumed that Clovis hunters came south via an ice-free corridor, through the glaciers that covered most of Canada until about 14,000 years ago. But researchers now realize that a number of sites in North

and South America predate 14,000 years, suggesting that the first Americans arrived before the Clovis people and before the ice-free land corridor opened up. Hence the renewed interest in the coastal hypothesis.

The paper itself sticks to the facts of the finds, with little speculation about their bearing on the coastal hypothesis. But Erlandson

and others think that the sites are clearly relevant. For example, although the new sites are radiocarbon-dated to no earlier than 12,000 years ago, back in 1959, 13,000-year-old human bones were uncovered on Santa Rosa Island at Arlington Springs. Thus the islands may have been occupied at least as early as the inland Clovis sites. Erlandson notes that the projectile points found on the Channel Islands look nothing like Clovis points, which were probably used to hunt large mammals. But they do resemble points found at pre-Clovis sites in the Pacific Northwest, South America, and even Japan, where similar artifacts dated to 15,000 years ago have been found.

Moreover, the team argues, the earlier finding of the Arlington Springs remains indicates that Paleoindians must have used boats by at least 13,000 years ago, suggesting that they had the seafaring skills needed for early coastal migrations. "The presence of people on the islands by that ... date in itself suggests they had the maritime capabilities to get there," says anthropologist Quentin Mackie of the University of Victoria in Canada.

But is the new evidence really proof of specialized coastal adaptations? "These appear to be strictly seasonal settlements of highly mobile hunter-gatherers, focused on shellfish and bird harvesting," says Yesner, who adds that such forays could have been carried out with "relatively primitive" boats. And David Meltzer of Southern Methodist University in Dallas, Texas, wonders whether "this represent[s] a full-blown marine adaptation of a bunch of seafaring people cruising down the coast, or did they wander in" from inland areas, bringing the stemmed points and crescents with them.

So what would it take to prove the coastal hypothesis? "Unequivocal evidence would be a pre-Clovis site located on the coast," says Beck. Meltzer agrees: "Give me a site" on the coast that is at least 15,000 years old, "and I will be a happy guy."

Finding such a site is a tall order, however, because sea levels have risen nearly 100 meters over the past 15,000 years and most coastal sites are now submerged. Some archaeologists have begun searching for them using underwater techniques. Archaeologist Daniel Sandweiss of the University of Maine, Orono, says ancient DNA analyses of Paleoindian bones might also help (<http://scim.ag/childburial>). To settle the debate, "we need to find where the bodies are," he says.

—MICHAEL BALTER

CREDITS (TOP TO BOTTOM): UNIVERSITY OF OREGON; JON M. ERLANDSON

ORNITHOLOGY

Feathers Are Flying Over Colombian Bird Name Flap

Last May, at the Washington, D.C., home of the Colombian ambassador, the American Bird Conservancy (ABC) and its partner in Colombia, Fundación ProAves, announced the discovery of a new species of Neotropical bird. ABC touted the feat as “remarkable” for being one of the first times a new species had been scientifically described from an individual captured, measured, photographed, and then released. For George Fenwick, head of ABC, it was a proud moment: The bird, Fenwick’s antpitta (*Grallaria fenwickorum*), was named in honor of his family.

The problem with the announcement was that it made no mention of the bird’s actual discoverer, a 28-year-old former employee of ProAves named Diego Carantón, and the two preserved specimens he had already collected. How Carantón, his specimens, and the name he had chosen—*Grallaria urraoensis*—came to be omitted from the taxonomic record is generating bitter debate between Colombia’s leading university ornithologists and ProAves, the country’s best-known private conservation organization, over scientific standards and credit for new discoveries.

ProAves’s leadership, in a lengthy statement expected to be released this week, says Carantón violated his employment contract by secretly collecting specimens, so the foundation was justified in rushing out a paper last May to seize nomenclatural priority—accorded to whoever publishes first—and name the bird after Fenwick.

ProAves agrees that Carantón identified the new species. In 2008, while Carantón managed ProAves’s Colibrí del Sol reserve in the Western Andes, he found a wet, unrecognizable bird dead in a capture net. He could not find the bird in any museum collections and eventually captured, and killed, a second bird. “I knew ProAves doesn’t like collecting, but I did it. I wanted to know what the bird was,” Carantón says.

Carantón also did not immediately tell ProAves about the unfolding discovery, and foundation officials were furious to learn not only that there were two specimens already in a drawer at Bogotá’s Institute of Natural Sciences but also that Carantón had involved other researchers, including Colombia’s leading avian DNA expert, Daniel Cadena of the University of Los Andes, in the project. “It was a cross, a double-cross, a triple-cross for ProAves,” says David Caro, ProAves’s



All in a name. Rival groups have described and named this Colombian antpitta, one from the live bird and the other from dead specimens.

outgoing executive director.

Initially, Carantón was given permission to publish a description of the new species on the condition that he use the name *G. fenwickorum*. But negotiations became embittered as ProAves sought final say over the paper and the list of authors. “All the intellectual property of the discovery belongs to ProAves, and we have every right to authorize a publication or not, considering the bird was discovered on our reserve,” read one e-mail that Sara Lara, currently director of international affairs for ABC, sent to scientists and ProAves staff.

To make matters worse, the name *G. fenwickorum* didn’t sit well with the scientists. In 2008, Fenwick had penned an editorial sharply critical of Colombian museum collectors, accusing them of gratuitously killing endangered bird species. Cadena later resigned from the project, explaining in an

e-mail, “I definitely don’t want to be part of a homage to Fenwick.”

When Carantón eventually decided to publish the discovery independently—and name the bird *G. urraoensis*, after the local municipality of Urrao—ProAves countered by hosting a hastily arranged field expedition. In May 2010, two junior field guides scooped Carantón, publishing a description of *G. fenwickorum* in ProAves’s in-house magazine, *Conservación Colombiana*, grabbing the right to name the bird. “It came down to the name,” says Caro. “We needed the name to raise more funds.”

The paper held another dart for the academics who had sided with Carantón. Given Fenwick’s opposition to killing rare birds for science, ProAves had collected only a few feathers and photographs—materials that in a pinch can be accepted as the museum holotype, or reference specimen. However, ►

many collection curators believe a full specimen is needed to create an accurate record. For F. Gary Stiles, curator of the bird collection at the National University of Colombia in Bogotá, the attempt to designate the feathers and photographs as the holotype is “scientifically irresponsible.”

ProAves’s publication also caused dissent inside the foundation’s leadership. “I thought it was just too much to steal a young man’s discovery of a lifetime that way,” says ornithologist Niels Krabbe of the University of Copenhagen, one of two ProAves board members to resign in protest.

Two months later, a description by Carantón and a co-author appeared, this time in the journal of the Colombian Association of Ornithology. They called the bird *G. urraoensis*. In a lengthy editorial, Cadena and Stiles said ProAves had probably won naming priority but charged “grave faults” in the organization’s scientific ethics.

Carantón’s defenders argue that Carantón has a “moral right” to his discovery, despite any missteps. They call the case the latest in a series of hard-boiled moves by ProAves aimed at gratifying British and American donors and generating publicity at the expense of Colombian researchers. “They have created a lot of tension in the country,” says ornithologist Luis Germán Naranjo, who is conservation director for the World Wide Fund for Nature in Colombia.

There is little question that ProAves has been successful in protecting birds. Since its founding in 2001, the organization has bought 22,000 hectares of Andean habitat, creating 18 reserves for 91 threatened species. That land includes some of the only known habitat of the Colorful Puffleg, a hummingbird, and ProAves is credited with a resurgence of the rare Yellow-eared Parrot.

Success has brought a list of over 100 mostly foreign donors, including the U.S. Fish and Wildlife Service and ABC. Paul Salaman, conservation director at World Land Trust in Washington, D.C., and an influential ProAves board member, says “the meteoric rise” of ProAves may have generated resentment. “It wants to get things done, and that upsets people,” says Salaman.

Some researchers still hope *G. urraoensis* could win out before international bodies that rule on species names. However, Ellinor Michel, executive secretary of the International Commission on Zoological Nomenclature in London, says it will be difficult to unseat *G. fenwickorum*. “If the publication by ProAves [was] legitimate, then the name they gave will stand,” she says.

—ANTONIO REGALADO



ARCHAEOLOGY

Ten Years After Buddhas Destroyed, Afghans Work to Save Monastery

First they fired artillery. Then they exploded bombs. Ultimately, the Taliban’s destruction of the world’s two largest Buddha statues, which began a decade ago this week, required explosive charges up and down the vast niches holding the statues. The Buddhas had stood watch over central Afghanistan’s Bamiyan Valley for some 1500 years, until the Taliban finally demolished them in March 2001. This week, cultural heritage officials met in Paris to review years of work to stabilize the fragile niches and to prepare for the spring opening of an open-air museum at the site.

Even as they acknowledge this notorious anniversary, Afghan archaeologists are focusing on another Buddhist complex 200 kilometers south of Bamiyan that stands in peril of destruction, called Mes Aynak. The threat here is not Taliban intolerance but development: Mes Aynak’s giant mounds of monasteries happen to sit atop the world’s second largest deposit of copper. The government and a Chinese mining company have agreed to delay operations, to avoid an embarrassing replay of Bamiyan. But to preserve Mes Aynak’s treasures will require a huge international rescue effort—perhaps the largest archaeological endeavor ever undertaken in Afghanistan, says Brendan Cassar, the UNESCO cultural heritage officer in Kabul. Plans for the project face time, money, and security constraints in the war-torn country.

“Cultural heritage in Afghanistan is still under threat 10 years after Bamiyan,” adds Cassar. He and others say that although there have been successes—such as renovating the

National Museum in Kabul and recovering important artifacts—Afghanistan still lacks funds and expertise to secure ancient sites and disrupt looting networks.

The Bamiyan anniversary and next week’s opening of the first exhibit on the finds at Mes Aynak, at the National Museum, are putting the spotlight on Afghanistan’s beleaguered heritage. Once thought to be isolated outposts, monasteries such as those at Mes Aynak and Bamiyan now appear to have been large and complex centers that played a crucial role in linking India, Central Asia, and China. At Bamiyan, the destroyed Buddhas—38 and 55 meters high, respectively—were the most dramatic part of a monastic community that included a network of caves filled with exquisitely detailed paintings. Many were destroyed before and during the Taliban’s rule, though Japanese experts are conserving the remainder. Mes Aynak, a half-hour’s drive south of Kabul, has never been excavated but included 19 ancient settlements within a 40-hectare area, says Philippe Marquis, head of the French archaeological mission based in Kabul, who recently completed a detailed survey.

A Chinese company was awarded a \$3.5 billion contract to mine copper at Mes Aynak—the largest such contract in the country’s history. It intended to begin mining last year despite the unexcavated archaeological riches. Following an international outcry (*Science*, 30 July 2010, p. 496), the Afghan Ministry of Mines agreed in September to allow researchers time to excavate the area

CREDIT: SHAH MARA/AFP/GETTY IMAGES/NEWSCOM



Gone, but not forgotten. A massive niche stands empty at Bamiyan (*left page*). Philippe Marquis (*above in beret*, at Mes Aynak) and Omar Sultan (*far right*) struggle to preserve what's left of Afghanistan's heritage.

and conserve the hundreds of fragile statues and wall paintings before operations begin. "We will have 3 years to excavate the site," says Omar Sultan, deputy minister of culture in Kabul.

Sultan, an archaeologist, was the one who first spotted the importance of Mes Aynak in the mid-1970s. But excavators began probing the site only in 2009, shortly after the mining contract was awarded. They quickly began to uncover a dazzling array of richly decorated monasteries and related settlements that flourished from the early centuries C.E. until after the arrival of Islam in the 7th century C.E., including wall paintings and large reclining, sitting, and standing Buddhas, as well as chapels, stupas, and dormitories.

Like Bamiyan, Mes Aynak was a center of learning that helped spread Buddhism throughout Central Asia and into China, says art historian John Huntington of Ohio State University in Columbus. The monasteries also appear to have played important economic and political roles. The monks and residents at Mes Aynak, for example, mined copper for profit. Buddhism was thought to have been snuffed out with the arrival of Islam, but increasing evidence suggests that the two faiths coexisted, with Hinduism and Zoroastrianism, for centuries.

Researchers say a thorough excavation of Mes Aynak will be a Herculean effort that could require \$45 million and 1000 workers. A multimillion-dollar 10,000-square-meter conservation facility is slated to be built nearby, with funding provided by the U.S. government, according to officials in Kabul. Marquis says the World Bank has agreed to

provide \$4 million for initial work, that the International Security Assistance Force, based in Kabul, may also contribute, and that the Chinese government recently agreed to provide archaeologists to help. Given the sheer size and scope of the project, however, "we may need more time beyond 3 years," says Marquis. "It's not a hell of a lot of time to excavate a site of that extent," adds Vienna University art historian Deborah Klimburg-Salter, who visited the site late last year and was stunned by its size and obvious importance. Omara Khan Masoudi, who directs the National Museum in Kabul, fears the ticking clock. "If we lose the artifacts, we lose our heritage," he says.

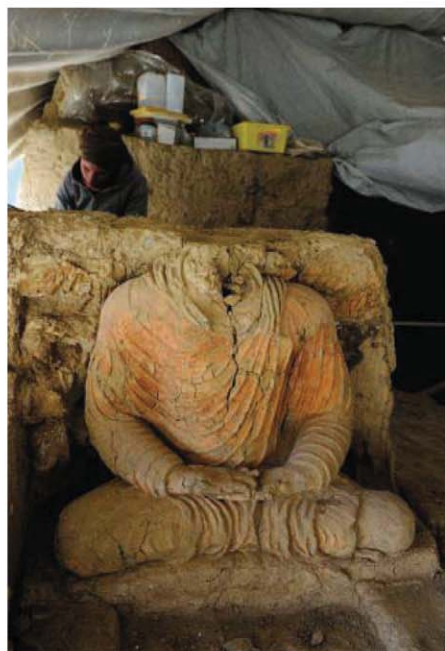
Already, security fences are in place around the rugged site, which lies in a Taliban-friendly area. Nearly 2000 Afghan guards reg-

ularly patrol the boundaries, and a camp for Chinese workers has been built in the center, enclosing one of the monasteries partially excavated in 2009. But smelters and a railroad to ship the copper to China are still lacking, and one official close to the project says that given the world economic downturn, the company may be in no rush to begin copper extraction. Mining will require destruction of the site, officials say, so archaeologists must decide what is worth rescuing before those operations begin.

The early results of the Mes Aynak digs will be on display at the first public exhibition of the site's materials, which will open on 15 March at the National Museum. "We advocate an extension of the museum to house the new material from sites like Mes Aynak," says Cassar. A U.S. embassy source says the American ambassador may use the Mes Aynak opening as an occasion to pledge "a significant commitment" to the museum.

Meanwhile, back at Bamiyan, Cassar says proposals to reconstruct the massive Buddhas were rejected as expensive and impractical. Conservators have instead spent \$5 million in the past 7 years fixing dangerous cracks in the niches, removing unexploded mines and bombs from under the statues' rubble, and conserving what they can of the fragments for display. Ironically, the Buddhas' destruction left piles of pegs, timbers, and other elements that provide new insight into how the massive structures were built. A German-led team has dated fragments of the statues to the 6th century C.E., later than scholars once thought, and confirmed that the Buddhas were brightly painted—the larger with orange robes, the smaller in white (<http://scim.ag/Bud-dhas>). The story of their creation, along with that of the destruction, will be laid out in the modest open-air museum slated to open as early as this spring in the remote valley northwest of Kabul.

—ANDREW LAWLER



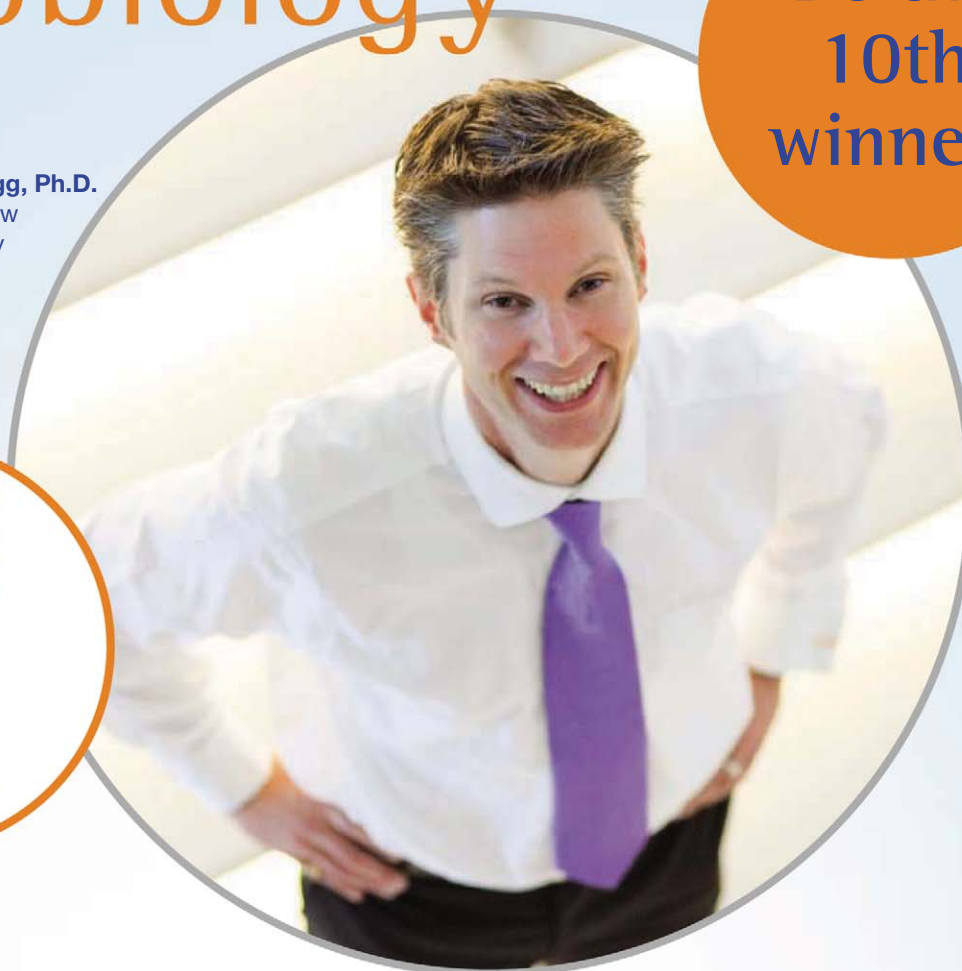
Troubled treasures. This Buddha from Mes Aynak shows the ancient monastery's sophistication.

CREDITS: (TOP, LEFT TO RIGHT) A. LAWLER; PASCAL LE SEGRETAIR/GETTY IMAGES; (BOTTOM) SHAH MARAI/AFP/GETTY IMAGES/NEWS.COM

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Congratulations to Dr. Christopher Gregg on winning the 2010 Eppendorf & Science Prize for his studies on genes that alter their expression in the brains of offspring according to whether they were inherited from the father versus the mother. His findings suggest new pathways that may help to understand brain diseases such as autism, schizophrenia and eating disorders.

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To be eligible, you must be 35 years of age or younger. If you're selected as this year's winner, you will receive US\$ 25,000, have your work published in *Science* and be invited to visit Eppendorf in Hamburg, Germany. Past winners and finalists have come from as far a field as China, Chile, India and New Zealand. Yes, it *can* happen to you!

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SCIENCE EDUCATION

Outrage Greet NSF Decision to End STEM Fellows Program

Researchers are shocked and upset by a decision by the U.S. National Science Foundation (NSF) to cancel a high-profile and successful fellowship program that has brought more than 10,000 graduate students into elementary and secondary schools around the country. A recent evaluation says the Graduate Science, Technology, Engineering and Mathematics (STEM) Fellows in K–12 Education program, begun in 1999, brings science to life for students, improves the skills of their teachers, and offers graduate students valuable training in the classroom. So participants don't understand why the president's 2012 budget request would abandon a \$55-million-a-year program that addresses key aspects of the Obama Administration's push to improve U.S. science and math education.

NSF officials say that the GK-12 program, as it is commonly known, has served its purpose and that some of its features will be incorporated into other NSF programs that train graduate students and that partner academic scientists with local school districts. "GK-12 has been effective, but much of it is now being done by other programs," says Joan Ferrini-Mundy, head of the agency's education directorate. "NSF doesn't fund things forever. It was time to take what we had learned and move on, applying those lessons to other settings."

But scientists who have received GK-12 training grants aren't satisfied with that explanation and have launched a campaign to save the program (savegk12@gmail.com). No other program, they say, puts graduate students into the classroom and creates a unique learning opportunity for students, their teachers, and the fellows themselves.

Responding to Ferrini-Mundy's comment, Susan Williams of the Bodega Marine Laboratory at the University of California, Davis, asks rhetorically, "Where are we going to move to? GK-12 is irreplaceable. It addresses the pipeline issue in a way that no other graduate training program does." Williams, who is the principal investigator for a GK-12 grant to improve ocean literacy among high school students, says that "having young scientists as role models can make a lasting impact on students, and the program is large enough to have a real impact on K–12 STEM education."

Win-win-win situation

Rita Colwell launched the GK-12 program shortly after becoming NSF director (Science, 29 October 1999, p. 895). The

Graduate Research Fellowship

Begun: 1952

Purpose: To support graduate students pursuing degrees in all fields that NSF funds and to encourage the best basic research.

Current budget: **\$136 million**

Integrative Graduate Education and Research Traineeship (IGERT)

Begun: 1998

Purpose: To support innovative new models for graduate training, fostering collaborative research that transcends traditional disciplinary boundaries.

Current budget: **\$70 million**

GK-12

Begun: 1999

Purpose: To support graduate students in K–12 classrooms, improve teacher content knowledge, build ties between university researchers and local schools.

Current budget: **\$56 million**

Math and Science Partnership

Begun: 2002

Purpose: To support innovative partnerships to improve K–12 student achievement in mathematics and science.

Current budget: **\$58 million**

CANCELED

Not so jolly fellows. The GK-12 fellows program is slated for extinction in NSF's stable of programs that support graduate students.

program, which gives out 5-year grants that typically support eight to 10 students a year, grew rapidly, reaching \$50 million by the time Colwell left in 2004. It was such a favorite that it was even highlighted in the agency's budget requests to Congress.

NSF operates many programs that support graduate students (see graphic), and its \$900-million-a-year education portfolio also contains an ever-evolving assortment of efforts aimed at teachers. NSF routinely assesses that portfolio, so in 2006 it hired an outside contractor to evaluate the GK-12 program. The review by Abt Associates of Cam-

bridge, Massachusetts, delivered last fall, cited "substantial and credible evidence that the program has clearly been able to achieve many of its goals."

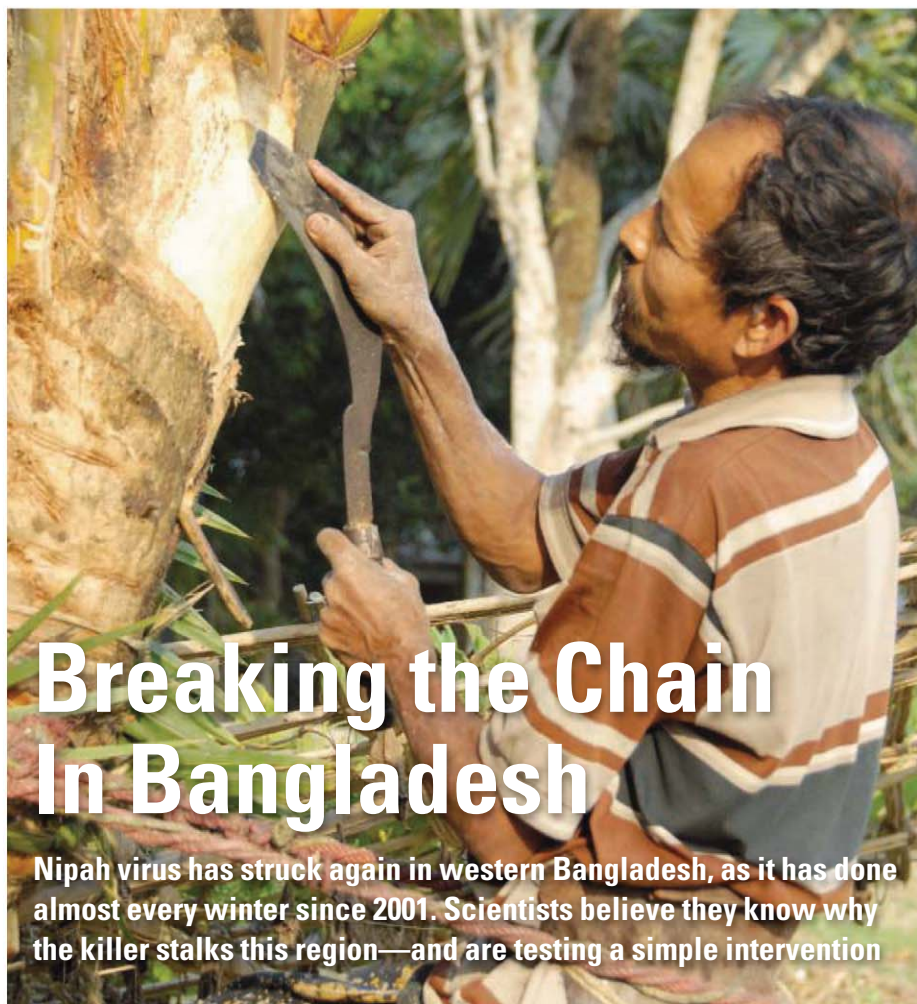
The report found that everyone involved seemed to be benefiting. The fellows became better teachers, learning how to work collaboratively and how to communicate science to a nontechnical audience. The public school teachers improved their knowledge of science and welcomed having graduate science students in their classrooms. The fellows' new skills made them better college instructors, and their off-campus experiences gave them an edge in finding full-time jobs after graduation.

The evaluation also found that the fellows did not take any longer to complete their degree, something that many faculty members had feared. The program's only real weakness, according to Abt, was its modest impact on the students' research skills.

NSF officials cite that "mixed" result in explaining why they decided not to make any new awards, although the agency will honor its commitment to current grantees. Time-tested NSF programs like the much bigger Graduate Research Fellowships do a better job of training graduate students to become researchers, according to Ferrini-Mundy, and newer ones such as its Math and Science Partnership program also give entire university departments a chance to interact with local schools. There also may be better ways to raise student achievement, says Carl Wieman, associate director for science within the White House Office of Science and Technology Policy. "The reality is that, under GK-12, the time available for kids to interact with grad students so that both sides could develop a relationship ... was probably not enough to achieve the impact that one would have liked to see," says Wieman.

Colwell, now a distinguished professor at the University of Maryland, College Park, and at Johns Hopkins University Bloomberg School of Public Health in Baltimore, Maryland, says she thinks it is "unfortunate" that NSF has decided to end the program. "It's been extraordinarily successful. Of course there are other programs for graduate students. But this is unique in using them to bring science into the K–12 class at the same time it supports their graduate education. And given the poor state of STEM education in the schools, this is vitally important."

—JEFFREY MERVIS



Breaking the Chain In Bangladesh

Nipah virus has struck again in western Bangladesh, as it has done almost every winter since 2001. Scientists believe they know why the killer stalks this region—and are testing a simple intervention

RAJBARI, BANGLADESH—When Anwara Begum came down with a high fever on 28 December, she and her husband thought it was a cold or the flu. As the hours passed, she grew too weak to eat or get out of bed, and she couldn't move her fingers. On the 30th, Begum's husband, Ramjan Ali, took her to a clinic, where doctors administered fluids for her diarrhea and discharged her. By the time she got home, she was unable to speak. "Both of us were very scared," says Ali.

Early in the morning on 1 January, Begum died. Three days later, her 2-year-old daughter, Dilruba, started running a fever. The toddler grew more and more lethargic, then could no longer move her limbs. Before doctors at Faridpur Medical College Hospital could move her to an isolation ward, Dilruba was dead. The hospital sent blood samples to the Institute of Epidemiology, Disease Control and Research (IEDCR) in Dhaka, the capital. An antibody test confirmed the worst: Dilruba had succumbed to Nipah virus, a rare pathogen discovered in 1999. IEDCR dispatched a team to probe how she and her mother contracted the disease and to scour

Rajbari here in western Bangladesh for more cases. The evidence uncovered heightens the mystery about why this region has become ground zero for Nipah.

Nipah claims few lives, but in South Asia it is a dreaded malady. The virus kills almost three-quarters of those it infects in Bangladesh, where nearly all known cases in the past decade have occurred, and leaves many survivors with crippling neurological disorders. "This virus is a bad actor. It causes a striking degree of anxiety and panic," says epidemiologist Stephen Luby of the U.S. Centers for Disease Control and Prevention (CDC), who has been on assignment with the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) in Dhaka since late 2004. Nipah's hallmark symptom is brain inflammation, or encephalitis. "Previously healthy young people die and die in groups," says Luby. "This really is a community crisis."

Nipah first emerged in Malaysia in 1998, but only in Bangladesh has it become a peren-



Risky business. A *gachhi* taps a date palm in western Bangladesh. Bamboo skirts (above) appear to prevent bats from contaminating collected sap.

nial scourge. Almost every winter since 2001, the virus has flared up in the world's most densely populated nation. It's more brush-fire than inferno: All told, the virus has killed 111 people in Bangladesh during the past decade. Fifty people died in 2004, the worst year. This winter is shaping up to be bad, with the death toll at 27 as *Science* went to press—and 2 months to go in the Nipah season.

Nipah is also a scientific puzzle. Disease hunters believe they have pinned down the virus's natural reservoir—fruit bats—and they have nailed a transmission route in Bangladesh: consumption of contaminated date palm sap. But some things don't add up. Fruit bats test positive for Nipah antibodies across southern Asia, and date palm sap is a delicacy throughout Bangladesh. Yet the virus mostly haunts only what investigators call "the Nipah belt," a clutch of districts near the Ganges River in western Bangladesh. "There is something about this area, at this time of year, that puts people at particular risk," says Luby.

A gnawing fear is that Nipah virus will break out of its geographic box. IEDCR Director Mahmudur Rahman is optimistic that won't happen. "I believe that we will be able to keep the disease under control," he says. "But if we fail, it will be a real disaster for the country."

Out of hiding

Nipah first garnered attention in September 1998, when pigs on farms in Malaysia started

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S Podcast interview
with author
Richard Stone.



Caught in the act. *Pteropus* fruit bats (top) are the natural reservoir for Nipah virus. Infrared cameras have observed *Pteropus* and other bats licking sap from tapped date palms.

getting sick in droves. Public health authorities assumed it was swine fever: The pigs had classic symptoms, such as high fever, muscle spasms, and abnormal gait. But it differed in one way: Many pigs developed loud barking coughs. Piglets readily succumbed to the disease, but most grown pigs recovered.

Then farmers started getting ill—and dying. Their symptoms included high fever, muscle pain, and severe encephalitis. Malaysian health authorities assumed the killer was Japanese encephalitis (JE), a mosquito-borne virus—pigs are a reservoir—and ordered widespread pesticide fogging. Pig handlers were vaccinated and told it was safe to go back to work. But some of them came down with encephalitis and died, too. Still convinced that they were dealing with JE, health officials assumed that batches of the inactivated vaccine were ineffective and turned to a live-attenuated JE vaccine. Another red herring was that some victims tested positive for JE antibodies. These may have been false positives, says Kaw Bing “Paul” Chua, a virologist at Temasek Life Sciences Laboratory in Singapore, or else pig farmers had been exposed to JE in the past without becoming ill.

In February 1999, Chua, then at the University of Malaya in Kuala Lumpur, joined the investigation when a patient with acute encephalitis was admitted to the university’s medical center. His serum also tested positive for JE antibodies. But anti-JE interventions appeared to be having little or no

effect, so Chua suspected an unusual or novel pathogen—and went fishing. He inoculated cell lines with cerebrospinal fluid and serum from their patient and five others. The pathogen ravaged Vero cells from African green monkey kidney tissue, making them fuse and form syncytial cells. That’s the calling card of paramyxoviruses, a diverse family that includes measles virus, mumps virus, and respiratory syncytial virus. Chua bombarded the Vero cells with monoclonal antibodies against several paramyxoviruses. Nothing stuck, which suggested that the virus might be new to science.

The University of Malaya’s aging electron microscope produced only blurred images of the viral particles. On 12 March, Chua packed samples in dry ice and flew to the United States. At CDC’s laboratory in Fort Collins, Colorado, microbiologist C. Bruce Cropp put the samples under their electron microscope, and when the image appeared, Chua immediately recognized the ringlike structures of paramyxovirus nucleocapsids. “Great fear overwhelmed me,” he says. Paramyxoviruses, he knew, are spread by close contact, via saliva or sputum. No wonder the JE control measures had failed. A few weeks later, Chua and CDC collaborators identified a novel paramyxovirus.

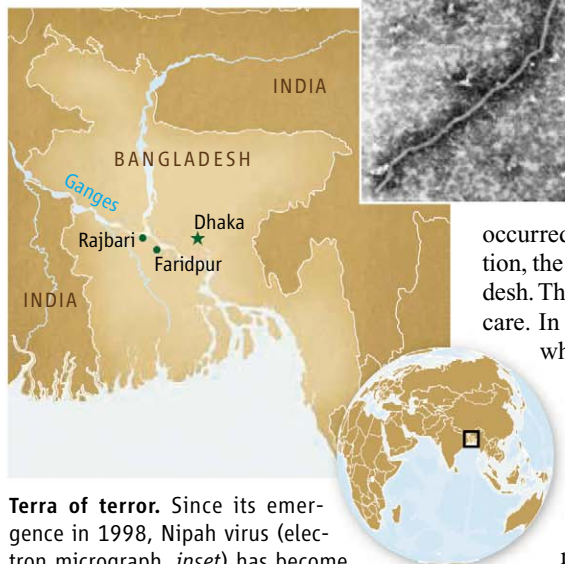
Malaysian authorities quickly changed tack. They ordered a mass cull of some 1 million pigs and sought to limit human contact with pigs to break the transmission chain. The measures worked. Through June 1999 the virus—named Nipah, after the village where it was first isolated—had infected 265 people in Malaysia and 11 pig slaughterhouse workers in

Singapore, killing 106. The tally does not include several encephalitis victims in 1997 whose stored sera were later found to have anti-Nipah antibodies, says Chua.

The virus was no longer infecting pigs or humans—but it had to be hiding somewhere. During the outbreak, Nipah virus had also been isolated from other livestock and domestic animals like cats and dogs. But its natural reservoir was unknown.

Scientists had a good lead though. Nipah’s closest kin is Hendra virus, which emerged in the mid-1990s in Australia. That virus had infected several horses and their handlers, causing an illness marked by severe encephalitis that killed two people. Scientists in Australia found live Hendra virus in *Pteropus* fruit bats, commonly called flying foxes, and concluded that this species is the virus’s natural reservoir. In Malaysia, researchers uncovered Nipah antibodies in scores of fruit bats roosting in trees near the pig farms where the outbreak had occurred. The bats would eat durian flowers and mangoes and other fruits in groves near pig farms; presumably their Nipah-tainted urine or saliva infected the pigs, says Chua. After months of searching, in early 2000 they found live virus in one bat from a large colony on Tioman Island, off peninsular Malaysia’s east coast. The team declared that it had uncovered Nipah’s reservoir; some experts were dubious. “There was skepticism about the single isolation from Tioman,” says Luby.

For 2 years, Nipah virus laid low before reemerging far from the original epicenter—and with a whole new modus operandi. Western Bangladesh, 2500 kilometers northwest of Malaysia, experienced its first outbreak in early 2001. This time, pigs had nothing to do with disease transmission. Victims were contracting Nipah from an unknown source, and it was spreading from person to person—something that rarely occurred in the Malaysia outbreak. In addition, the mortality rate was higher in Bangladesh. The likely explanation is inferior health care. In Malaysia, many acutely ill patients who were put on ventilators pulled through. Bangladesh has only a handful of ventilators, and to date, only one patient has been put on one, Luby says. “This is a terrible virus,” he says, “but I don’t think there’s something qualitatively more virulent about the strains we’re seeing here compared to the strain that was seen in Malaysia.”



Terra of terror. Since its emergence in 1998, Nipah virus (electron micrograph, inset) has become a recurring nightmare only in western districts of Bangladesh.

A Startling Villain

DHAKA—When villagers in northeastern Bangladesh began dying 3 years ago, disease sleuths were stumped. “Usually we have a good idea of what we’re dealing with,” says Emily Gurley, an epidemiologist with the International Centre for Diarrhoeal Disease Research, Bangladesh, here. “This was not one of those outbreaks.” The surprising culprit they uncovered sends a chilling warning to regions facing food shortages.

Alarm bells rang on 4 November 2007, when a woman and her 3-year-old child were taken unconscious to a hospital in the Sylhet District, on the Indian border. Early that morning, relatives explained, an older daughter had died; all three had vomited and were restless. Within hours, the mother and younger girl were dead. More cases cropped up; because people were succumbing quickly and without a fever, says Gurley, “we suspected something toxic.”

After a few days, a common thread began to emerge. Victims had eaten a plant that locals call *gaghra shak*. University of Dhaka botanist Mohammad Zashim Uddin determined that villagers had consumed seedlings of common cocklebur (*Xanthium strumarium*). Scouring the literature, the scientists learned that livestock have died after grazing on these seeds and seedlings; there was one report of three children in Turkey who died after eating seeds.

Villagers in Sylhet said they commonly used leaves or stalks of mature cocklebur to flavor curries or for medicine. That’s fine, as the toxin, carboxyatractyloside, is present in large amounts only in seeds and seedlings. But that summer, monsoon flooding had reduced rice yields. Impoverished villagers resorted to eating cocklebur seedlings. “As the flood waters receded, these were the only plants that were sprouting,” says Gurley, lead author of a report on the outbreak in the March 2010 issue of *PLoS ONE*.

Scientists briefed villagers on the danger in town hall meetings; newspapers picked up the story, too. All told, 76 people took ill; of 19 deaths, 13 were children under age 10. The unforeseen consequence of food insecurity was a sobering lesson for Gurley. “I tried to imagine what it would be like to prepare a meal of weeds for my two young children because we had no food,” she says. “I was unable to imagine it.”

—R.S.



Taking their cue from Malaysia, researchers zeroed in on *Pteropus* fruit bats, which are common across Bangladesh. Hendra virus and Nipah virus are now classified as Henipaviruses.

Antibodies to Henipaviruses have been found in *Pteropus* bats across Southeast Asia and from as far away as Madagascar and the African continent. A few years ago, live Nipah virus was isolated from a fruit bat in Thailand, which has never reported a human case. In Bangladesh, scientists haven’t been so lucky. Working with colleagues from the nonprofit EcoHealth Alliance, Luby says, “We have identified an awful lot of bats that are antibody-positive. It’s just a matter of time until we find live virus.”

Bangladesh had a Nipah-free year in 2002. The virus returned in 2003 and struck with a vengeance in 2004. The outbreak that year in Faridpur “confirmed the substantial risk of person-to-person transmission,” Luby says. Meanwhile, Malaysia hasn’t reported a case since 1999. The only other country where Nipah is known to have jumped into humans is India. It has reported several sporadic cases and one outbreak, when the virus killed four dozen people in Siliguri, a city just across the border from Bangladesh, in early 2001.

That presented a riddle. The virus appeared to be endemic to a broad swath of Asia. “Fruit bats are everywhere,” Luby says, and have similar levels of Nipah antibodies wherever researchers have looked. It’s possible that Nipah flies under the radar elsewhere. In more than half of encephalitis deaths in Asia, says Luby, the cause is unidentified. “It’s plausible that sporadic cases are going unrecognized,” he says. Yet only western Bangladesh is hit with recurring outbreaks.

Russian roulette

When Badsha Shaikh was a child, he says he had a talent for climbing trees. Now he’s nearing 50 and still spry. A curved dagger tied to his waist, Shaikh scampers up a 20-meter-tall date palm almost like he is dashing straight up the trunk. “It’s like he’s running up stairs,” anthropologist Rebeca Sultana of ICDDR,B says with a laugh. In several seconds, he’s near the top and slings in place a leather harness. Shaikh is a *gachhi*, or date palm sap collector. During the winter months here in

Ramchandrapur village, when the sap is running sweet, Shaikh tends a couple of dozen palms; the rest of the year, he makes a living as a rickshaw driver.

Fruit bats once were common here, says Shaikh, but some locals enjoy eating them, so there are fewer now. In the afternoon, Shaikh shimmies up his date palms, slashing v-shaped cut marks in patches of trunk stripped of bark just below the fronds. The rest of the day and overnight, trees ooze sap through the wounds, which funnel the liquid into a clay jug slung below the bare patch. Just before dawn, Shaikh clambers back up to retrieve the jugs. Most sap will be boiled down to molasses in big kettles.

The best stuff is quaffed raw. *Gachhi* identify one or two date palms—older, taller, female trees—that produce the tastiest sap.

“It’s sweet. We used to drink it often when we went in the field, before Nipah,” says Sultana. Children love it mixed with puffed rice. “You have to drink the sap by 9 or 10 in the morning,” Sultana says. After that, it begins to ferment. Approximately 90% of the population in Bangladesh is Muslim, so consuming alcohol is forbidden. Some villagers imbibe fermented sap on the sly.

Drinking the stuff raw, it turns out, is a game of Russian roulette. During field investigations, IEDCR and ICDDR,B researchers discovered a common association with many Nipah cases not

explained by human-to-human transmission: The victims, both adults and children, had drunk raw date palm sap before getting sick.

Then the researchers caught bats in the act. In early 2008, a team led by ICDDR,B veterinarian Salah Uddin Khan trained motion sensor-tripped infrared cameras on tapped date palms. Over 20 nights, they made dozens of observations of *Pteropus* and other bats alighting on the shaved part of the date palms. Sometimes the bats would lick the sap running into the jug. Sap in the jugs, researchers surmise, can be contaminated with bat saliva and urine—and thus the virus. That may explain Nipah’s seasonality in Bangladesh. Cases here have occurred only between December and April, roughly coinciding with the time that palm sap is collected.

A second hypothesis is that seasonality is linked to the bat’s biological rhythms. Nipah virus and *Pteropus* bats appear to have



“This virus ... causes a striking degree of anxiety and panic.”

—STEPHEN LUBY,
CDC EPIDEMIOLOGIST



coevolved over centuries or millennia. “Bats don’t seem to be bothered by it,” says Luby. “It’s like *E. coli* in our guts.” Bats breed in cycles, and females give birth around the same time. “Presumably, the mother’s antibodies protect newborn pups from Nipah. Then the antibody wanes and they become susceptible,” says Luby. During that period, more virus might be shed in the urine, heightening transmission risk.

With strong evidence that date palm sap is an important transmission pathway, scientists and health experts are looking for ways to break the transmission chain. “Getting people to stop drinking raw date palm sap is the only answer,” argues IEDCR’s Rahman. That will be devilishly hard to pull off: Drinking raw sap is a cherished Bengali custom, says Sultana.

Scientists have hit upon a measure that may stem infections in the short term. Villagers told anthropologist Nazmun Nahar, now a consultant to ICDDR,B, that years ago *gachhi* would protect the part of the date palm trunk stripped of bark with a covering made from bamboo slats. These bamboo skirts had fallen out of use.

In 2007, ICDDR,B persuaded *gachhi* to deploy bamboo skirts on a few trees. Infrared cameras indicated that the skirts are effective. “They keep the bats out, so the sap is cleaner,” says Luby. This year, with support from the U.S. Agency for International Development, they are scaling up the intervention and trying jute, which can be fashioned into skirts more cheaply and quickly, and plastic. ICDDR,B staffers have been going from village to village to hold community meetings to explain the risk of raw date palm sap and the importance of using skirts. They persuade village elders to put up warning posters. “This may

not be a basic science question, but it’s the cutting edge of emerging infections,” Luby says. “How do you come up with solutions that are scientifically sound, locally acceptable, and can be scaled up?” Crucially, date palm owners are warming to the concept. “I can stop drinking raw sap, but it’s hard to stop my children from drinking it,” says Abdus Sobhan Shaikh, who is now using jute skirts on two of his 20 trees.

More widespread awareness of the threat might have saved Begum and her daughter. Ali told investigators that his wife had drunk raw date palm sap at least three times in December, before she became ill. Each time she shared the ambrosia with several other women—none of whom came down with the disease. That the virus infected only Begum and Dilruba, and spared their companions, makes theirs a classic case.

Heaven sent?

In Rajbari, ripe brown sapodilla fruits are hanging from the boughs of trees and rotting on the ground. “Fruit bats love it,” says Khan. In a village on the outskirts of town, three veterinarians wearing face masks soothe a calf tethered to a tree. It lows plaintively. One guy jabs a syringe into its jugular. The team is drawing blood from as many animals as they can lay hands on: livestock, dogs and cats, and, of course, fruit bats, which are captured with nets strung near rooks. A handful of Nipah cases in the past have been linked to sick cows and goats, and a cluster in 2003 was traced to a pack of nomadic pigs.

Khan’s team came here within a few days after Anwara Begum’s death to draw animal blood. Then they were looking for immunoglobulin M (IgM) antibodies, which develop within a few days after exposure to an antigen.

Coming to grips. Responding to an outbreak in Rajbari last January, a team led by Salah Uddin Khan (*top right*) draws blood from a calf to look for anti-Nipah antibodies. Rebeca Sultana (*bottom right*) and colleagues are going village to village warning about the risk of raw date palm sap.

Results are not yet in. The researchers have come back in early February, a month after Begum’s death, to prospect for immunoglobulin G antibodies, which show up in the blood after a few weeks.

In contrast to the Malaysian outbreak, which was triggered by one or possibly two strains, Nipah outbreaks here have involved a number of strains. “We see quite a bit more variability,” says Luby. Not surprisingly, infected individuals in Bangladesh exhibit a range of symptoms, with mild cases called “Nipah fever” and, compared with Malaysia, more cases with pneumonia. If some strains are more apt to infect the lungs, Luby says, that could explain why many Nipah cases here are spread person to person. Epidemiology can’t shine a bright enough light on such questions: The number of cases is too small, says Luby. “So we are aggressively seeking strains,” he says.

In western Bangladesh, the scars from Nipah run deep. Some survivors are never the same. “People suffer personality changes and cognitive deficits. In a number of cases, these deficits have not gotten better,” says Luby. And because there is no vaccine or drug against Nipah, when an outbreak occurs, villagers feel helpless. “Many people think, ‘Medical care can’t do anything; it must be supernatural.’ It looks like a curse from Allah,” Luby says. If his team is right, simply preventing date palm sap contamination may be all it takes to banish the curse. **—RICHARD STONE**

PARTICLE PHYSICS

Have Physicists Already Glimpsed Particles of Dark Matter?

The debate over that question suggests that the discovery of dark matter—whenever it comes—will be a murky affair

For decades, astronomers' observations have indicated that some elusive "dark matter" provides most of the gravity needed to keep the stars from flying out of the galaxies. In recent years, cosmologists' studies of the afterglow of the big bang, the cosmic microwave background, have indicated that dark matter makes up 80% of all matter in the universe. Now, many physicists expect that within 5 to 10 years they will finally discover particles of dark matter—that is, if they haven't already done so.

Data from three experiments all suggest that physicists have glimpsed dark matter particles much less massive than they had expected, or so argue Dan Hooper, a theorist at Fermi National Accelerator Laboratory in Batavia, Illinois, and his colleagues. Physicists working on other experiments say their results rule out such particles, but Hooper contends that a realistic look at the data and the uncertainties shows no fatal contradictions.

The case isn't conclusive, Hooper emphasizes. "I think it's fairly compelling," he says, "but we all agree that we're going to need something else to convince us that what we're seeing is dark matter." Still, Hooper's work has some physicists nervously tugging at their collars. "When I saw Dan's ... analysis I thought, 'Oh God, I better go back and take a second look [at our data] and make sure I didn't miss anything,'" says Peter Sorensen of Lawrence Livermore National Laboratory in California, who worked on an experiment that didn't quite have the sensitivity to test the idea.

Whether or not Hooper's claim stands up, the debate surrounding it underscores two characteristics of the search for dark matter. First, nailing down the particles' properties will likely require connecting many subtle and ambiguous clues. "To discover the nature of dark matter will take a village," says Rocky Kolb, a cosmologist

at the University of Chicago in Illinois. "I don't expect a eureka moment," when one decisive observation makes everything clear. Second, it's a particularly contentious field. With several relatively small teams (by particle physics standards) competing for a piece of a huge prize, researchers are cagey about discussing their results. And accusations of spinning the data to bolster one claim or another fly this way and that.

When seeking dark matter particles, physicists have three options. First, they can look for the particles floating by, as our galaxy supposedly lies embedded in a vast dark matter "halo." Dark matter particles should barely interact with ordinary matter, so such "direct searches" require sensitive detectors housed deep underground, where levels of cosmic rays and ordinary radiation fall but dark matter can penetrate. If a dark matter particle strikes an atomic nucleus, then the recoiling nucleus can produce a tiny pulse of electricity, light, and heat.

Second, scientists can turn their eyes to the skies. When two dark matter particles collide, they may annihilate each other, producing gamma rays or other familiar particles. "Indirect searches" might spot the annihilations by detecting excess gamma rays coming from places such as the center of our galaxy with instruments like the orbit-

ing Fermi Gamma-ray Space Telescope or the ground-based High-Energy Stereoscopic System (HESS) in the Khomas Highland of Namibia. Finally, an atom smasher might blast dark matter particles into existence in what's called an "accelerator-based search."

All three methods may soon pay off, physicists say. That hope is bolstered by a notion called supersymmetry that solves conceptual problems in particle theorists' "standard model" and posits for every known particle a massive undiscovered partner. Some partners could be weakly interacting massive particles (WIMPs) that would make ideal dark matter particles. And if supersymmetry is going to patch up the standard model, then those WIMPs should be observable, Kolb says: "Within the next 10 years, we'll either have very strong evidence of what WIMPs are or—because you can never kill an idea—we'll have given the idea a near-death experience."

But if Hooper is right, physicists may have already seen signs of such particles. In the past 6 months, he and his colleagues have laid out their case in four papers posted to the arXiv preprint server (www.arxiv.org). Their arguments rely on something old, something new, something borrowed, and something circling our blue planet.

The old is a controversial result from the Dark Matter (DAMA) detector in Italy's subterranean Gran Sasso National Laboratory. In 2000, physicists reported signs of dark matter particles striking nuclei in a 100-kilogram array of sodium iodide crystals to produce flashes of light. The rate of flashes varied over the year, peaking in June and bottoming out in December. That's what should happen if the galaxy spins in a dark matter halo so that the solar system faces a "wind" of dark matter particles blowing at 230 kilo-

meters per second. As Earth's orbit carries the planet into that wind, it should appear to blow 30 kilometers per second faster, increasing the rate of particle detections. The rate should fall as Earth swings away from the wind.

The team has now traced that signal for 13 years, the last few with the enlarged DAMA/LIBRA detector, and nobody doubts it's there. DAMA researchers say they cannot identify anything other than dark matter particles that could produce a signal like the one they observed. "Nothing has been found or suggested by anyone in over a decade,"



Vindicated? For a decade, physicists with the DAMA/LIBRA detector (*above*) have claimed an observation. New results may bolster their case, one theorist says.

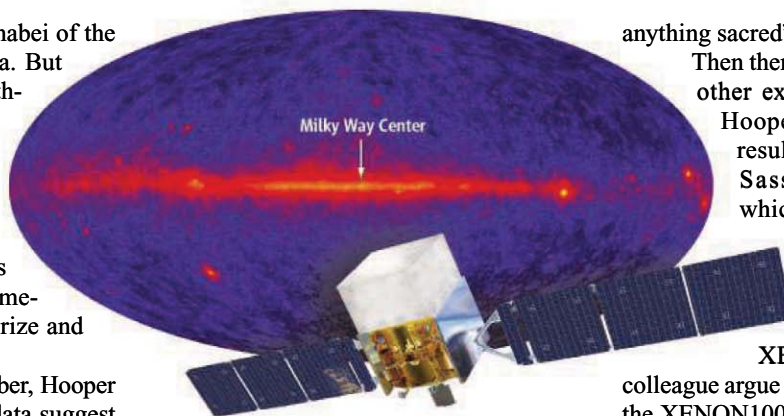
CREDIT: COURTESY OF R. BERNABE/UNIVERSITY OF ROME TOR VERGATA

says the team's leader, Rita Bernabei of the University of Rome Tor Vergata. But other experiments have seen nothing, and other researchers say the DAMA team hasn't always explained its crosschecks. "In the past, they've been pretty tightlipped with the details," says Livermore's Sorensen. "It's like they're saying, 'We saw something. Now give us the Nobel Prize and go away.'"

In a paper posted on 27 October, Hooper and colleagues argue that new data suggest DAMA may be seeing dark matter after all. The data were first presented at a conference in Marina Del Ray, California, in February 2010 by researchers working with the Coherent Germanium Neutrino Technology (CoGeNT) detector in the Soudan Underground Mine in northern Minnesota. The 440-gram cylinder of germanium produces an electrical signal when struck by a particle, and researchers saw a tantalizing excess of very low-energy events.

At first, many physicists thought the DAMA and CoGeNT results might agree. Their enthusiasm waned, however, when on a graph of particle mass versus strength of interactions with ordinary matter, the DAMA and CoGeNT results pointed to different regions, suggesting that they could not be signs of the same particles. However, the results depend on so-called quenching factors to relate a signal's size to the energy of the recoiling nucleus, and Hooper realized that the DAMA team used an average value with a tiny uncertainty. So he took from the literature a less-certain low-energy estimate—that's the something borrowed. "You really should include all the uncertainties," he says. "When you do, these regions get a lot bigger and overlap."

Then there are observations from the great blue beyond. In a paper posted on 31 December, Hooper and Lisa Goodenough of New York University examined publicly available data collected by the Fermi satellite, launched in June 2008, as it peered at the galactic center. The pair took into account emissions from the galactic disk and the broad bulge in its middle. They extrapolated from the higher-energy gamma ray spectrum mea-



Hot spot. Fermi (inset) may be seeing gamma rays from dark matter in the galaxy's core.

sured by HESS to estimate the spectrum at lower energies expected from the galactic center. But the measured flux of gamma rays exceeded expectations for energies below 7 billion electron-volts (GeV). That excess could signal dark matter annihilations.

Not everyone is convinced. In fact, Juan Collar of the University of Chicago, who leads the CoGeNT team, says the team isn't claiming a signal. "We're calling it a background," says Collar, a co-author on Hooper's October paper. Peter Michelson of Stanford University in Palo Alto, California, says Fermi researchers' own analysis of gamma ray emission from the galactic center shows an excess around 3 GeV. But so far it's too small and uncertain to be significant, he says. "The bar must be very high for claimed detections of dark matter," Michelson says. "We are not there yet."

The dark matter particles would not be exactly what many expected, either. The data point to particles weighing about 7 GeV, or seven times as much as a proton. Physicists expect WIMPs to weigh 10 times more. The

W in WIMP stands for the weak nuclear force through which the things would interact with ordinary matter. So WIMPs should weigh about as much as the particles that convey that force, the W and Z bosons, which weigh 80 and 91 GeV. Still, in papers posted 2 September and 5 November, Hooper and colleagues argue that a 7-GeV particle fits into established theory and supersymmetry. "They don't have to violate

anything sacred" to do it, Kolb says.

Then there is the question of whether other experiments already skewer Hooper's claim. In September, results from a detector at Gran Sasso called XENON100, which contains 170 kilograms of liquid xenon, seemed to rule out such particles. But Sorensen, who worked on the earlier XENON10 experiment, and a colleague argue in a 31 January preprint that the XENON100 limits rely on an untenable extrapolation of a quenching factor.

A more serious challenge comes from a preprint posted 10 November by physicists working with the Cryogenic Dark Matter Search II (CDMS II) experiment at Soudan. CDMS II detects pulses of electricity and heat when particles strike ultracold disks of silicon and germanium. Looking at just the heat signals to snare the lowest-energy events and analyzing the data conservatively, CDMS II rules out the particles' existence, says Blas Cabrera, the team leader from Stanford. "We believe it robustly rules it out," he says. "It's not even close." Hooper acknowledges that the CDMS II limit imposes a constraint but says "there's still wiggle room" for light dark matter particles.

The debate over Hooper's claim reveals the contentious nature of the field, in which pretty much every team draws fire for overstating what its data show. For example, Collar isn't always as conservative as he professes to be about interpreting CoGeNT's excess, Sorensen says: "He may tell you that it's a background, but let me tell you, when he gives a talk he doesn't try to dissuade theorists from interpreting it as a signal."

What would clinch the case for a light dark matter particle? Little doubt could remain if CoGeNT saw an annual cycle that matches DAMA's, Hooper says. But Collar won't be convinced of any sighting until the purported particles also emerge in collisions at the world's largest atom smasher, the Large Hadron Collider at the European particle physics laboratory, CERN, near Geneva, Switzerland. "If people don't see it in accelerator experiments and other ways, I'm just not going to believe it," he says.

Even if his claim falls apart, Hooper says he won't regret having made it. "I enjoy taking something that might be true and exploring it rather than working all the time on something I know to be true," he says. He's also had the pleasure of stirring up the community, which seems fairly easy to do with expectations running so high.

—ADRIAN CHO



"I think [the case is] fairly compelling, but we all agree that we're going to need something else to convince us that what we're seeing is dark matter."

—DAN HOOPER,
FERMI NATIONAL
ACCELERATOR LABORATORY



PROFILE: HELGA NOWOTNY

Keeping Europe's Basic Research Agency on Track

Helga Nowotny played a key role in the successful start of the European Research Council. As its president, the Austrian sociologist still has two major tasks ahead of her

VIENNA—There were a few times when Helga Nowotny considered throwing in the towel and abandoning the fledgling European Research Council (ERC) she'd helped create. The thought would typically arise when she couldn't bear the weight of the Brussels bureaucracy anymore.

In 2007, for example, the ERC's scientific council, of which she was then vice-chair, wanted to fly several hundred young applicants for the first round of grants to Brussels for interviews. Weeks before the invitations were to go out, the European Commission's (EC's) legal service said no: E.U. rules did not allow payment of E.U. travel money to grant applicants. "We never saw these lawyers," Nowotny says. "We called them the secret legal service. It was maddening."

The lawyers eventually caved in—but not until Nowotny and her fellow council members arranged a meeting with EC President José Manuel Barroso. The incident was just one of many frustrating episodes. Throughout the birth and the 4-year existence of the ERC, scientists' ideas on how to run a funding agency for creative frontier science have clashed with the EC's rules for managing an international bureaucracy. For the scientific council—a group of 22 heavyweights

from across Europe, which Nowotny now chairs—it has been a steep learning curve and a source of deep frustration. "We were naïve," Nowotny says. "We got caught in this web of rules."

Nowotny, a petite, 73-year-old sociologist of science with the energy of someone half her age, hung in there, and in most cases, eventually got her way. The ERC, which has disbursed almost €3 billion since 2007, has become popular with scientists and is considered a success story in European research policy.

But Nowotny—who works at an office close to the University of Vienna and Sigmund Freud's old apartment—still has two major tasks to accomplish before she steps down in 2013, at the end of the ERC's first 7-year mandate. One is an overhaul of the organizational structure that she hopes will put it on more solid ground and wrest power away from the officials and politicians of the EC. The other is a substantial hike in the ERC budget for the period from 2014 to 2020. Her opening gambit: a more than 200% increase from the current level.

Those are tall orders, but insiders say few are better placed to accomplish them than Nowotny. "She's extremely capable and she

has an excellent political sense," says Dutch physicist and E.U. science policy expert Peter Tindemans. "She's very well networked and very effective. At meetings, she's always going from person to person, talking, talking, talking," says Frank Gannon, a veteran of European science administration who now heads the Queensland Institute of Medical Research in Brisbane, Australia. Nowotny is "strong and powerful," he adds.

Midwife

Helga Nowotny was born and raised here, except for a high school year spent in Wisconsin, where she fell in love with 1950s American youth culture and learned to play the saxophone. Back in Vienna, she studied law, found a job at a criminology institute, and obtained her law degree in 1959. Then her husband's career took her to New York, where she studied sociology at Columbia University. Giants of the field like Paul Lazarsfeld—an Austrian Jew who had left Vienna in the 1930s—and Robert Merton were her teachers.

After getting her Ph.D. in 1969, she specialized in the sociology of science, a field also known as science and technology studies, or STS. She has co-authored more than a dozen books but is best known for her contribution to *The New Production of Knowledge*, a 1994 book written with British science policy analyst Michael Gibbons and others. Its thesis was that traditional science was being replaced by what the authors called Mode 2: research driven by applications and societal questions, less organized by discipline and hierarchy, but based on collaborations in flexible teams.

CREDIT: HEIMO AGA/SIPA PRESS

On the move. Nowotny has a busy travel schedule, but her office is in Vienna, where she was born.

The book was controversial; critics said Mode 2 had always existed, or that it wasn't clear whether it was an empirical description of reality or rather a model to follow. But the book cemented Nowotny's reputation as the "grand lady of STS," says sociologist Pieter Leroy of Radboud University Nijmegen in the Netherlands—even though her sharp pen has made her a few enemies as well.

After she retired as a professor at the Swiss Federal Institute of Technology in Zurich in 2002, Nowotny became one of the ERC's midwives. She was on the 2003 blue-ribbon panel that first concluded that Europe needed an agency for fundamental, curiosity-driven, bottom-up research. Until then, the E.U.'s Framework Programme (FP) primarily funded large collaborations, centering on applications and requiring groups from many E.U. countries. The ERC would be different because it would fund individual scientists and its sole criterion would be excellence.

Between 2001 and 2006, Nowotny chaired the 45-member European Research Advisory Board (EURAB), which issued several ringing endorsements of the idea. She kept on board EURAB's industry representatives, who were lukewarm, and helped to win over Philippe Busquin, then Europe's commissioner for research. "She speaks everybody's language," says French astronomer and former EURAB member Catherine Cesarsky. In 2005, Nowotny joined the embryonic ERC's scientific council as vice-chair; she took over as chair last year, when Imperial College London molecular biologist Fotis Kafatos stepped down. "She seems to be having her third youth," says Leroy.

Nowotny helped convince the council that the ERC should cover not just life sciences and physics but also the social sciences and humanities—an unusual concept in the Anglo-Saxon world. "We fund research in the 19th century, German conception of *Wissenschaft*, which includes everything," she says. She had proposed that 18% of the budget be spent on social sciences and humanities; the council rounded it down to 15%.

Her passion for the ERC stems in part from her feeling as a true European. She has lived and worked as a scientist in Hungary, Germany, France, the United Kingdom, and Switzerland. She moved back to Vienna in 2004—this time, for good, she says. She's still ambivalent about her hometown, how-

ever; she loves Vienna's quality of life and the arts scene but abhors its provincial, xenophobic streak.

A frenetic traveler, Nowotny says she often makes multipurpose trips, such as last month, when an old friend retired at The Hebrew University of Jerusalem. The university offered to organize an "ERC day," during which grantees presented their work and the Weizmann Institute of Science organized a dinner for her. She flies to Brussels once or twice a month, because despite the ERC's initial success, Nowotny isn't finished.

Tripling the budget

Overhauling the ERC's awkward organizational structure is important because it hampers the mission, Nowotny says. The ban on inviting applicants was just one example. EC rules demanded that grant reviewers, some of them world-famous scientists, fax in a copy of their passports to prove their identity. The scientific council wanted to give grantees credit cards for expenses; EC lawyers said it couldn't be done. The basic problem, according to a 2009 panel: The scientific council sets the policy but is dependent on the EC to get it executed (*Science*, 31 July 2009, p. 523).

The situation has definitely improved, Nowotny says. Since last year, day-to-day management rests with a so-called Executive Agency, a structure in Brussels that operates at arm's length from the EC and is more flexible. Nowotny praises its "highly professional" staff members and says working relationships are much better now. But a world-class funding agency can't be dependent on the goodwill of civil servants, she adds.

That's why, at Nowotny's request, Máire Geoghegan-Quinn, the current research commissioner, has set up a task force to devise a new structure within the E.U.'s tight constraints. Reforming the Executive Agency is one option, but lawyers are also studying a

paragraph added to the E.U. Treaty last year stipulating that the union "shall establish the measures necessary for the implementation of the European research area." It might allow abandoning the agency and starting something entirely new. The task force is chaired by Robert-Jan Smits, the EC's director general for research, whom Nowotny finds easy to work with and understanding of the ERC's needs.

Her other big job is securing a permanent budget hike. The ERC was allotted just €7.5 billion out of the €51 billion total for FP7, which spans 2007–13. The amount actually spent is ramping up as the years go by and will reach €1.7 billion by 2013. Nowotny wants at least double that—€3.4 billion per year—from 2014 on. That would be €24 billion over the entire 7-year period of FP8, a tripling of the budget. Nowotny knows that's a lot to ask for from the EC, but she was delighted when Geoghegan-Quinn called herself "probably the ERC's greatest fan" in a recent interview with *Science* (18 February, p. 844).

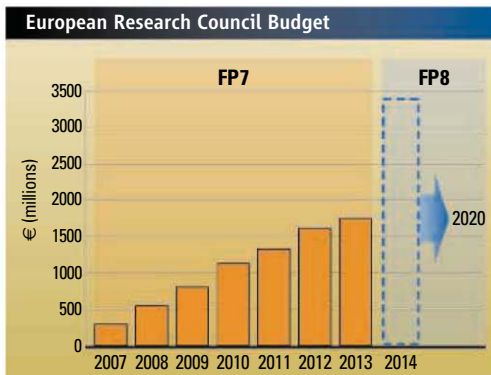
Still, given the economic downturn and the fiscal crises in several European countries, Gannon says he believes that such a drastic increase is unlikely to happen. He cautions that countries that fare less well in the ERC's competitions—which are mostly in eastern and southern Europe—may start wavering in their support. To keep them involved, he has suggested that the ERC create a special competition, still using excellence as a criterion but aimed at countries that spend less on science.

That's anathema to Nowotny. Other parts of the Framework Programme address the needs of lagging countries, she says; besides, the ERC is a stimulus for countries to reform their universities and make them more competitive. "Behind closed doors, politicians and scientists in those countries tell me: Don't change the system," she says.

For similar reasons, she's adamantly against affirmative action for women, despite their embarrassing lack of success at the ERC. Of the latest round of Advanced Grants, for established scientists, 9.4% went to women—down from 15% in 2009. An ERC "gender equality plan," published earlier this month, promises some mitigating steps, such as encouraging women to apply for grants and ensuring that review panels are balanced.

But what Nowotny won't do—as much as the skewed sex ratio pains her—is lower the bar for women. "It would go against the ERC's core principles," she says. "We can't do it." And given her strength and power, that's likely to be the last word.

—MARTIN ENSERINK



Poised for a boost? Nowotny hopes that after Framework Programme 7 ends in 2013, the ERC budget will double to at least €3.4 billion per year.



LETTERS

edited by Jennifer Sills

Assessing Chemical Risk: Societies Offer Expertise

THE EFFECT OF ENVIRONMENTAL EXPOSURES ON HUMAN HEALTH IS A GROWING AREA OF concern. The number of new chemicals is increasing exponentially, with approximately 12,000 new substances added daily to the American Chemical Society's CAS registry (1). Although only a portion of these chemicals are introduced into the environment, data on the hazard posed by even high-production volume (HPV) chemicals (those with a production volume exceeding 1000 tons/year) are available for only a fraction of the HPV chemicals produced or imported

into the United States. Currently, the EPA and FDA are charged with safeguarding the health of Americans. This is a daunting task that is hampered by the growing recognition that currently accepted testing paradigms and government review practices are inadequate for chemicals with hormone-like actions.

The need for swifter and sounder testing and review procedures cannot be overstated. Recent scientific evidence has established direct links between exposures that occur during fetal development and adult disease [e.g., (2, 3)]. Data from the U.S. Centers for Disease Control and Prevention have established clearly that most, if not virtually all Americans, are exposed to con-

taminants in the environment that cause serious health effects in animal models [e.g., (4, 5)]. Direct links to humans remain uncertain, but there is sufficient experimental evidence to raise concern. Furthermore, there is growing evidence that some chemicals once thought to be safe and allowed into common and, in some cases, abundant commercial use may not be as benign as previously assumed (4, 5).

Although chemical testing and risk assessment have long been the domain of toxicologists, it is clear that the development of improved testing guidelines and better methods of assessing risks posed by common chemicals to which all Americans are exposed requires the expertise of a broad range of scientific and clinical disciplines. Collectively, our societies represent approximately 40,000 research scientists and clinicians. The membership of our societies represents leaders in the fields of reproductive biology, endocrinology, reproductive medicine, genetics, and developmental biology. As concerned scientists and clinicians, we are writing to offer the expertise of our collective societies. Specifically, we offer the expertise of the Boards of Directors for our societies for the purpose of naming appropriate individuals to serve on panels to review and evaluate current programs for effectiveness, to assess the risk of specific chemicals through the evaluation of data, and to develop new testing guidelines and protocols.

We recognize that the FDA and EPA face challenges on many fronts, and we believe that the vast expertise available through the members of our societies can aid both agencies in achiev-

ing their goals. Thus, we ask that you use our scientific boards to provide access to leading scientists in diverse fields. These experts can help ensure that the most up-to-date scientific methodology and scientific understanding are used when devising and refining regulatory guidelines, and when reviewing scientific data pertinent to risk assessment and risk management decisions.

**THE AMERICAN SOCIETY OF HUMAN GENETICS,
THE AMERICAN SOCIETY FOR REPRODUCTIVE
MEDICINE, THE ENDOCRINE SOCIETY,
THE GENETICS SOCIETY OF AMERICA,
THE SOCIETY FOR DEVELOPMENTAL BIOLOGY,
THE SOCIETY FOR PEDIATRIC UROLOGY,
THE SOCIETY FOR THE STUDY OF REPRODUCTION,
THE SOCIETY FOR GYNECOLOGIC INVESTIGATION**

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A Defense of the Primitive Cheetah Skull

IN THE NEWS FOCUS STORY "ALTERING the past: China's faked fossils problem" (R. Stone, 24 December 2010, p. 1740), Tao Deng of the Institute of Vertebrate Paleontology and Paleoanthropology in Beijing claimed that the cranium of a primitive cheetah (*Acinonyx kurteni*) that we described in 2009 (1) was "a fake" and that our conclusions were "based on a fossil forgery." These serious allegations were presented without tangible evidence. They were based not on personal study of the specimen,

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Human health. The effects of exposure to chemicals in food, air, water, and other products remain unclear.

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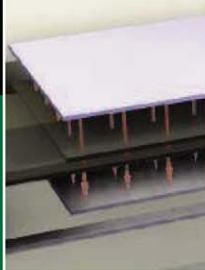
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Chromatin dynamics
and cancer

1145



Peeling graphene
layer by layer

1146

but on a few images in our study. It is true that the occipital area and zygomatic arches have been heavily restored in plaster, probably to make it appear more complete, thus enhancing its commercial value, a common malpractice among Chinese fossil dealers. We indicated this in our study. This does not impinge on *A. kurteni*'s status as a primitive cheetah; the cranium and dentition present a plethora of cheetah apomorphies, conclusively proving its membership of the cheetah lineage, but also several primitive traits hitherto unknown for cheetahs. The occipital area and zygomatic arches have none of these apomorphies. Deng claims that "the skull is a composite"—i.e., a chimera. In our original study, we found no evidence of this.

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Response

WE ARE PLEASED TO SEE THAT MAZÁK AND Christiansen note that the occipital area and zygomatic arches of the cheetah skull (1) have been heavily restored in plaster, as we claimed in the News Focus story. We stand by our view that the skull is a composite with fabricated features.

TAO DENG* AND ZHANXIANG QIU

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Letters to the Editor

Letters (~300 words) discuss material published in *Science* in the past 3 months or matters of general interest. Letters are not acknowledged upon receipt. Whether published in full or in part, Letters are subject to editing for clarity and space. Letters submitted, published, or posted elsewhere, in print or online, will be disqualified. To submit a Letter, go to www.submit2science.org.

Habitats at Risk: A Step Forward, a Step Back

TROPICAL FORESTS AND CORAL REEFS ARE well known as bastions of biodiversity, and Southeast Asia contains large areas of both. However, these habitats are threatened not only by human activity but also by climate-induced increases in sea temperatures and drought severity (1, 2). The steps we take to mitigate further degradation may be essential to saving these imperiled habitats. Here, we contrast positive steps taken by Thailand with insufficient action by Indonesia and the possible results of each course of action.

Sharp increases in sea temperature recently triggered widespread coral bleaching in reefs along the entire western length of the Malay Peninsula, from the tip of Sumatra to Myanmar (3). In response, the Thailand government closed several popular dive sites spread across seven Marine National Parks in an attempt to prevent damage by tourists (4), despite the risk of immediate losses to the tourism industry. Coral reefs may be particularly vulnerable to human disturbance during bleaching events (5), and Thailand's decision to take action may ultimately save this valuable natural resource. Bleaching events in the region are expected to become more common in the future (6), and preventative measures like the one implemented by Thailand will be crucial to ensure the survival of this endangered habitat.

Recent forest fire episodes in the region can also be partially attributed to climate change. Increased frequency of El Niño events and associated droughts in Southeast Asia have led to increased frequency of fires that destroy millions of hectares of tropical forests and peat swamps, particularly in Indonesia (7). These fires also produce large amounts of smoke that pollute the surrounding region, causing billions of dollars worth of losses in agriculture, timber, and tourism industries and contributing to

serious health problems (8). In the extreme 1997 El Niño year, fires from Indonesia produced the equivalent of 13 to 40% of mean annual global carbon emissions from fossil fuels (9); these enormous carbon discharges make Indonesia the world's third largest producer of greenhouse gases (10). Despite these threats, the Indonesian government's attempts to prevent these fires and the resulting smoke have been largely ineffective. As recently as October 2010, smoke blanketed the region, producing the worst haze in the region in 4 years (11).

Thailand's protective response to the latest coral bleaching event should serve as a model for other countries in the region and beyond. In contrast, Indonesia should work more actively to alleviate the increasing fire hazards in its forests and peat swamps. Some immediate steps that Indonesia could take include providing financial incentives to local communities to limit burning [e.g., through the United Nation's Reducing Emissions from Deforestation and Forest Degradation Programme (12)], providing alternative methods to clear land without using fire, and ratifying the Association for Southeast Asian Nations (ASEAN) Agreement on Transboundary Haze Pollution to engage neighboring countries and develop strict limits on the use of fire and joint plans to combat large fires (8). Climate change mitigation efforts have been far from satisfactory across the tropics, and Thailand's efforts should inspire others.

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MEDICINE

Trailblazer for Patient-Centered Care

Harlan Krumholz

Once in a generation or so, someone makes a fundamental discovery that, although obvious in retrospect, was met by successive stages of disinterest, resistance, and finally acceptance as a contribution that forever changed our view of the world. In medicine, through his discovery and thorough explication of ubiquitous small area variation, John Wennberg became one such individual.

In *Tracking Medicine: A Researcher's Quest to Understand Health Care*, Wennberg (Dartmouth Medical School) chronicles his journey for a wide readership. For the uninitiated, the book will serve as a primer on some of the most important work in the science of health care delivery. For those already familiar with Wennberg's work, the book offers a useful compendium of the evolution of his

inauspiciously sandwiched between a report on the influence of birth order on intelligence and a news item on the Watergate tapes. Their paper, a long time coming and having a somewhat critical view of clinical practice, had been previously rejected by many of the premier medical journals. With this contribution, modestly titled "Small area variations in health care delivery," the authors pioneered a field of research that has since transformed our views of the medical profession and health care delivery systems. In *Tracking Medicine*, Wennberg takes us along on his journey from his early revelation of inexplicable differences in the use of health care among regions in close proximity and with similar populations to his current research and views that are now influencing health care reform.

The book is elegant and disruptive. Wennberg highlights the power of enlightened observation as he identifies Vermont as the perfect laboratory in which he would demonstrate the frequently illogical nature of the delivery of health care. He describes populations that receive markedly different types of care despite similar characteristics and similar degrees of illness. He reveals that the likelihood of children undergoing a tonsillectomy was dictated by the doctor who treated them, a circumstance that in turn was based on the area where the children lived. And the differences he uncovered were not small. Wennberg relates how there could be as much as a threefold difference in rates between adjoining school districts. His work showed that although residents of Randolph and Middlebury were very similar and contacted their physicians at much the same rate, hospitalization and surgery rates were 66% higher (and Medicare spending 53% higher) for patients living in Randolph. Wennberg postulated that supplier-induced demand was responsible for these differences, and he went on to extend his work across conditions and regions to support his thesis. He comments that "variations that cannot be attributed to illness or access to care should be interpreted as an indication of variation in professional opinion, and the remedy for that variation ought to be outcomes research."

Wennberg also describes his work cham-

pioning the democratization of the patient-doctor relationship. He holds that "[i]t should be considered a serious form of medical error when surgeons operate on patients who would not have wanted the procedure had they been fully informed and empowered to participate in a meaningful way in the choice of treatment." His research exposed the shortcomings of approaches to health care in which the power resides solely with the physician. He

has shown the extraordinary effect of engaging patients with critical information. He describes his framework of preference-sensitive care, in which decisions for discretionary tests and procedures reflect not only the evidence for benefit but the patient's preferences. Wennberg comments that a "distinguishing feature of our

approach to outcomes was the insistence we placed on obtaining information about *all of the outcomes that matter to patients.*"

The author does not share his experiences on a personal level but rather as a recollection of an intellectual journey. Nevertheless, readers will feel his determination, creativity, disappointment, defiance, and confidence. Given the accomplishments it records, the book could easily be considered a capstone to a career—but I suspect that Wennberg is not finished.

Wennberg is generous to individuals who preceded him and to those with whom he collaborated. However, his account includes little description of the contemporaneous research performed outside his circle beyond that for purposes of contrast (as he does with the views of Robert Brook of RAND) or defense (as he does with the views of Peter Bach of Memorial-Sloan Kettering). This circumscribed focus is less a weakness than it is the expression of a sole author's detailed and unique narrative. Rather than offering a comprehensive history of the field, the book describes the steps and insights of its influential author. Nearly 40 years ago, Wennberg's keen observations about the delivery of health care in the United States launched a revolution in American medicine. *Tracking Medicine* tells the story that precipitated that revolution and should be required reading for anyone interested in how we can better provide health care—and how research can influence practice and policy.

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Listening to the patient.

thinking and the development of the research over his career. For both these audiences as well as readers who are already experts in outcomes research, Wennberg's account provides an inspiration about how outstanding research is conducted and a reminder of the persistence that is required to introduce new ideas and overcome conventional wisdom. At times technical, *Tracking Medicine* challenges readers to follow the author's trail, understand and interpret the details, and appreciate the meaning of his observations (including the graphical presentations of data that are sprinkled throughout the book).

In 1973, Wennberg and biostatistician Alan Gittelsohn published a remarkable research article in *Science* (1), which appeared

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Tracking Medicine

A Researcher's Quest to Understand Health Care

by John E. Wennberg

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ECOLOGY

The Biodiversity and Ecosystem Services Science-Policy Interface

Charles Perrings,^{1*} Anantha Duraiappah,² Anne Larigauderie,³ Harold Mooney⁴

In recognition of our inability to halt damaging ecosystem change (1–4), the United Nations Environment Programme (UNEP) was asked in December 2010 to convene a meeting “to determine modalities and institutional arrangements” of a new assessment body, akin to the Intergovernmental Panel on Climate Change (IPCC), to track causes and consequences of anthropogenic ecosystem change (5). The “blueprint” for this body, the Intergovernmental Platform on Biodiversity and Ecosystem Services (IPBES), lies in recommendations of an intergovernmental conference held in the Republic of Korea in June 2010: the Busan outcome (6). But it is a blueprint for governance rather than science. Using the experience from past assessments of global biodiversity and ecosystem services change (1, 7, 8) and from the IPCC (9–11), we ask what the policy-oriented charges in the Busan outcome imply for the science of the assessment process.

The Busan Outcome

Although previous global-change assessments required policy relevance, we argue that the policy orientation and support role of IPBES goes further. For example, rather than investigating consequences of specific policies identified by a governing body, most previous assessments were constructed around scenarios devised by scientists. The Busan outcome imposes a greater obligation on the IPBES to support specific policies, with implications for both the way the governing body gives charges to scientists and the way scientists carry out their work.

We focus on four functions identified in the Busan outcome: (a) “identify and prioritize key scientific information needed for policymakers at appropriate scales,” (b) “perform regular and timely assessments

of knowledge on biodiversity and ecosystem services and their interlinkages, which should include comprehensive global, regional, and, as necessary, sub-regional assessments and thematic issues at appropriate scales and new topics identified by science,” (c) “support policy formulation and implementation,” and (d) “prioritize key capacity-building needs to improve the science-policy interface....”

Three broad implications emerge: (i) The governing body of IPBES, the plenary, should ask for assessment of consequences of specific policies and programs at well-defined geographical scales. (ii) Projections of changes in biodiversity and ecosystem services should take the form of conditional predictions of the consequences of these policies and programs. And (iii), capacity-building efforts should enhance skills needed for policy-oriented assessment within IPBES and should catalyze external funding for underpinning science and science-based policy development.

Strengthening Policy Relevance

A critical lesson from the Global Biodiversity Assessment (GBA), the Millennium Ecosystem Assessment (MA), and the IPCC is that assessments should evaluate consequences of real policy options. This requires closer integration of the different elements of the science-policy process—research, monitoring, assessment, and policy development (12). Research uncovers mechanisms that explain how biodiversity change impacts ecosystem services and human well-being. Monitoring records trends in indicators of change. Assessment reports scientific evidence of change and evaluates mitigation, adaptation, or stabilization options identified by policymakers. Policy selects the “best” response. Although the establishment of IPBES means that all of these elements of the process will now be in place, all need to be strengthened if the new body is to discharge its functions effectively. Assessment, however, will be its core business.

The IPBES plenary will set the scale, focus, and terms of reference of assessments, although for preliminary assessments of emerging issues, this could be delegated to

Assessments must provide conditional predictions of the consequences of specific policy options, at well-defined spatial and temporal scales.

the executive to make it possible to respond in a rapid and flexible manner. To connect policy and assessment more closely, the terms of reference given to working groups should include the mitigation, adaptation, and stabilization options on which future projections are to be based. Examples might be the options identified by the Convention on Biological Diversity (CBD) in its 2020 targets (13, 14) or UNEP’s Green Economy initiative (15). The plenary will also establish a rigorous review process to assure the technical content of assessments and will be expected to retain direct responsibility for evaluating policy implications.

These functions have implications for the membership of the plenary. Although core membership will be national representatives, the issues that IPBES will be asked to address include many covered by multilateral agreements between nation states. The plenary will accordingly include representatives of multilateral agreements related to biodiversity and ecosystem services. As the primary conservation agreement, the CBD will be a natural and important member. However, it is only one among many relevant multilateral agreements. IPBES should support the other conservation conventions, as well as the many agreements dealing with ecosystem services (e.g., the UN Fish Stocks Agreement) or the drivers of change (e.g., the General Agreement on Tariffs and Trade).

These functions also have implications for the balance of disciplinary expertise required. This should span the natural and social sciences and should be reflected in IPBES leadership (the plenary and working group co-chairs), working groups (the scientists invited to carry out assessments), and the secretariat (the “permanent” IPBES staff scientists supporting assessments).

Strengthening Assessment

Matching assessments to policy needs affects the type of assessments undertaken. Some impacts of biodiversity and ecosystem change are local, others are national, regional, or global. Some are extremely fast, others occur on time scales more comparable with climate change. The policy focus of IPBES suggests that something like the sub-

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subsidiarity principle of European environmental policy (16) should apply. This principle, partially reflected in the MA global and sub-global assessments, holds that management of environmental problems should be at the scale consistent with capturing all relevant effects. We suggest that assessments should be undertaken at the smallest geographical scale consistent with capturing all relevant effects of the biophysical and social processes involved. Similarly, effects that occur on short time scales should be assessed more frequently and over a shorter time horizon than effects that occur on longer time scales.

Quantitative projections of impacts of global change on biodiversity—MA; UNEP Global Environment Outlook 4 (GEO-4); CBD Global Biodiversity Outlook 3 (GBO-3)—represent a major step forward for biodiversity assessment (17). In addition, assessments will need integrated models of social and environmental change that are capable of providing conditional predictions (probabilistic projections conditional on specific policy options identified by the plenary). This requires a step change in our capacity to model interactions between the socioeconomic system and the biophysical environment. Without an understanding of the feedbacks between the social and biophysical systems, it is not possible to assess the outcome of actions designed to alter the likelihood of environmental change (mitigation) relative to those designed only to alter its cost (adaptation) or to reduce stress on the uncontrolled parts of the system (stabilization).

There is also a case for strengthening monitoring activities. We anticipate that future assessments will call on the Global Earth Observing (GEO) Biodiversity Observation Network (BON), currently being designed as part of GEOSS, the Global Earth Observing System of Systems (18). Despite some focus on ecosystem services, however, GEO BON does not broadly integrate socioeconomic observations. Such data would be valuable to IPBES, and GEO BON has convened a working group to consider including such observations.

Strengthening Science

As with other assessments, IPBES will be charged with undertaking only synthetic and meta-analytical research, not the original research on which synthesis or meta-analysis is based. Although it is important to strengthen assessments, including the tools of synthesis, all assessments are ultimately only as strong as the supporting science. The MA, for example, was able to record physical changes in ecosystem services, but not

the value of those services. Yet that is what is needed for policy. The recent assessment of the economics of ecosystems and biodiversity (TEEB) shows that understanding of the social importance of changes in biodiversity and ecosystem services is still very patchy (19). In part, this reflects the fact that national funding for science is both highly uneven and biased toward national issues.

The four global change programs under the auspices of the International Council for Science (ICSU)—DIVERSITAS, the International Geosphere-Biosphere Program (IGBP), the International Human Dimensions Program on Global Environmental Change (IHDP), and the World Climate Research Program (WCRP)—will be natural partners of IPBES. But these also rely on national sources for research funding, and so reflect the same unevenness as national research efforts. Broadening and deepening the funding base for the research on which assessments of biodiversity and ecosystem services are made is a basic requirement for the success of IPBES (20, 21).

The Busan outcome recognized that to build capacity in basic science will require resources from elsewhere (e.g., development assistance budgets and governmental and nongovernmental research training funds). A key function of IPBES will be to catalyze such resources. One mechanism proposed in the Busan outcome is a “dialogue” between key scientific organizations, environmental policy bodies, and research funding organizations. We consider this a critical feature to address both scientific capacity and the policy relevance of research.

Conclusions

There is growing consensus that solving problems posed by global environmental change requires coordinated international research, better resourced than in the past, and paying at least as much attention to social science as it does to natural science (22, 23). The establishment of IPBES provides an important link with international policy, but its effectiveness depends on the quality of the underlying science. Knowing whether the effects of biodiversity and ecosystems services change are contained within a decision-maker’s jurisdiction is critical to the development of coordinated or cooperative management of the problem across jurisdictions. Knowing likely consequences of alternative policy options is critical to the choice of the best strategy.

For IPBES to provide the policy support envisaged in the Busan outcome, it needs to answer questions that are meaningful to the nations that have brought it into being.

This requires an approach that differs from those adopted in previous assessments—in the functions and membership of the plenary, in assessment methodology, and in decision support. The IPBES plenary should specify the policy options to be evaluated; assessment should include quantitative conditional prediction of the consequences of those options; and reports should enable policy-makers to evaluate the relative merits of mitigation, adaptation, and stabilization strategies. This requires a much higher level of commitment to capacity-building and to engagement with the policy community. The establishment of IPBES offers a unique opportunity to build on what has been done already. It should not be wasted.

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DEVELOPMENTAL BIOLOGY

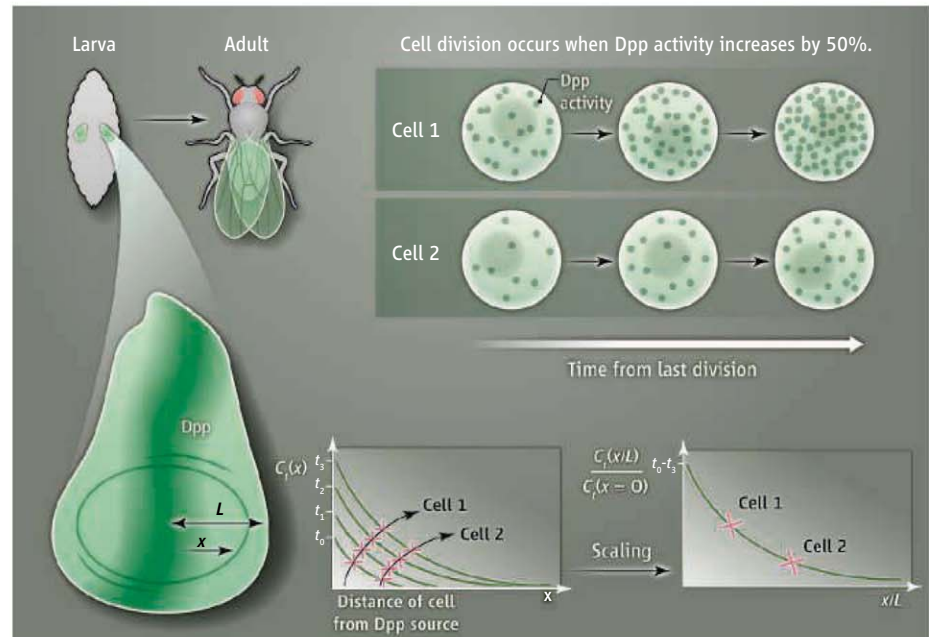
Gradient Scaling and Growth

Loïc Le Goff and Thomas Lecuit

What determines the final size of an animal? Secreted molecules called morphogens control tissue and organ growth during development (1). As morphogens diffuse away from their source, a concentration gradient forms. Target cells “read” the local concentration and activate genes involved in differentiation. One such morphogen is Decapentaplegic (Dpp), a member of the transforming growth factor- β family that controls fly (*Drosophila melanogaster*) development (2–6). Paradoxically, although Dpp forms a concentration gradient, it promotes seemingly uniform growth across its target tissue. Earlier studies argued that the slope rather than the concentration of the Dpp gradient may control growth (7), or that cell proliferation is modulated by mechanical constraints (8, 9). On page 1154 of this issue, Wartlick *et al.* (10) propose instead that cells control growth by computing the relative temporal variation in Dpp activity.

Wartlick *et al.* draw on both quantitative observations and theory to make three fundamental observations about the dynamic nature of the Dpp morphogen gradient. The first is that the gradient scales with tissue size, an issue that has long been unresolved. The authors examined the Dpp gradient in the *Drosophila* larval wing imaginal disc (which develops into the adult wing). By measuring the intensity of fluorescent proteins attached to Dpp and Dad (a molecule whose expression is induced by Dpp), the authors show that Dpp concentration and signaling activity are well described by an exponential function. The decay length (λ) of this exponential increases over time, proportionally with the size (L) of the tissue, such that λ/L is constant (see the figure). Thus, Dpp concentration profiles at all stages of development can be described by one unique exponential master curve.

Considering that growth is spatially uniform—that is, the relative position of a cell in the tissue remains unchanged—Wartlick *et al.* conclude that all cells in the tissue experience the same relative temporal changes in Dpp signaling. Thus, as a cell is moved



Uniform growth. The secreted molecule Dpp is produced from source cells in the developing fly larval wing tissue and diffuses across target cells to control growth. Dpp concentration profile, $[C_D(x)]$, for different times (t) of wing disc development is shown. x is the distance over which the gradient decays; L is the size of the target tissue; t_0 to t_3 represent different times during development. “+” indicates the concentration sensed by two cells (cell 1 and cell 2) as the tissue grows. In the scaled graph, these remain as stationary points. The two cells divide at the same rate because they experience the same relative increase in Dpp signaling.

away from the Dpp source because of tissue growth, its Dpp concentration does not decrease, but rather, it increases in proportion to gradient amplitude (maximum at the source) due to the accumulation of Dpp.

The third observation of Wartlick *et al.* is that the gradient amplitude is related to the area of the target tissue by a power law—they are proportional on logarithmic scales. Because all cells in the tissue sense the same relative temporal variation in Dpp, this finding implies that the rate of tissue growth, and hence the rate of cell divisions, is proportional to the relative increase of Dpp concentration and activity. Putting this into numbers, a cell divides when its Dpp concentration and activity increase by, respectively, 40 and 50%. Wartlick *et al.* hypothesize that this phenomenological correlation underlies a deeper causal link, and propose that sensing such an increase in Dpp signaling triggers cells to divide.

The quantitative model of Wartlick *et al.* lends itself to rigorous experimental testing. In particular, it predicts that any experimental manipulation of Dpp gradient dynam-

Tissue growth is controlled by the temporal variation in signaling by a morphogen along its concentration gradient.

ics should be accompanied by a change in growth rate, such that cells divide when Dpp activity has increased by about 50%. Indeed, even in perturbed conditions (such as altering the production, diffusion, or degradation of Dpp), cells at all positions in the imaginal disc tissue divide after experiencing a 50% increase in Dpp activity.

The correlation works so well that one might fear a “hidden” constraint embedded in the system that couples Dpp activity increase and tissue growth. To address this possibility, Wartlick *et al.* examined a situation in which the rise of Dpp activity is regulated externally by controlling the rate of accumulation of a constitutively active Dpp receptor, and thus the extent of relative Dpp signaling change in the target cells. The rate of cell growth correlated with the induction of cell division by a ~50% increase in Dpp signaling.

The model presented by Wartlick *et al.* changes how we should now think about growth control by morphogens in several ways. It establishes Dpp as a genuine growth factor, a point debated until recently. (Whether the effect of Dpp on early fly wing develop-

ment is mediated by the Fat-Hippo signaling pathway is possible but is not addressed in this study.) And it establishes a mechanistic link between the cellular computation of Dpp signal activity over time and cell growth. However, although the authors show that the model holds up to different experimental challenges and numerical simulations, the existence of a causal relationship is not firmly demonstrated. Direct observations, at the single-cell level, of cell division in response to an increase (by about 50%) in its Dpp activity should bolster the model.

The findings by Wartlick *et al.* point to new avenues of investigation. One question is how Dpp signaling activity is inte-

grated over time, and the form in which this information is stored and measured in the cell (11). Another concerns how polarized cell divisions in some regions of the wing imaginal disc could affect homogeneity of tissue growth and consequently the temporal variations in Dpp signaling that cells experience. The authors report the intriguing observation that the degradation rate of Dpp is inversely proportional to tissue size and decreases over time, thereby potentially explaining gradient scaling. This suggests that tissue growth affects Dpp dynamics. The existence and mechanism of this feedback will be an important problem to address in the future.

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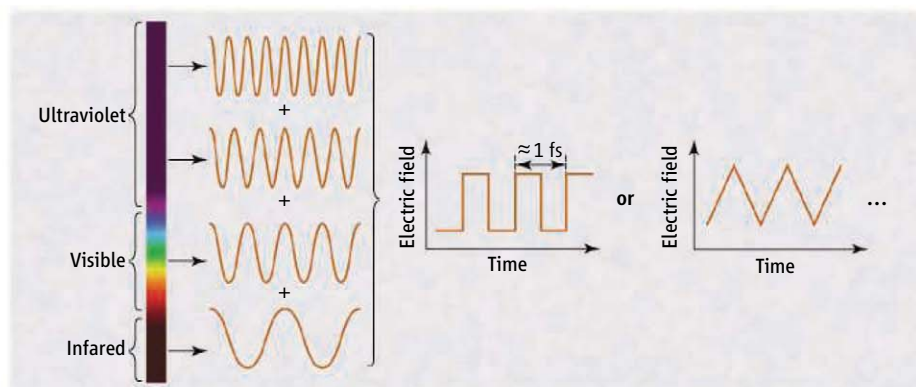
PHYSICS

Toward Synthesis of Arbitrary Optical Waveforms

Deniz D. Yavuz

Waveform generation underlies the operation of many electronic devices, especially those used in broadcasting and signal processing. Electronic waveform generators produce a prescribed series of voltages or currents as a function of time, which may then be used as an input for a variety of circuits. The simplest versions of these devices, known as function generators, are used in undergraduate classes to produce the familiar sinusoidal, square, or sawtooth voltage waveforms seen on oscilloscopes. Arbitrary waveform generators (called synthesizers) that can create almost any pulse shape are now a common piece of equipment (and often used by graduate students to test misbehaving electronic equipment). By comparison, synthesizing optical waveforms, in which the electric and magnetic fields of light waves are not simply oscillatory but are specified functions of time, has proved to be difficult. This task has been a long-standing goal of physicists since the invention of the laser in 1960 provided a source of coherent light. On page 1165 of this issue, Chan *et al.* (1) demonstrate an important step toward synthesizing and characterizing arbitrary waveforms in the optical domain.

The key difference between electronic and optical synthesizers is the time scales of these devices. Electronic synthesizers are



Arbitrary can be good. An ideal arbitrary optical waveform generator will produce coherent light covering visible, infrared, and ultraviolet spectral regions. Appropriate superposition of light waves at different colors can create arbitrary waveforms, for example, square and sawtooth waveforms.

generally limited to nanosecond (10^{-9} s) time scales, or frequency scales of 1 gigahertz. Optical synthesizers need to produce waveforms with time scales of 1 femtosecond ($1 \text{ fs} = 10^{-15} \text{ s}$), and frequency scales of 1 petahertz (PHz), so processing must be faster by six orders of magnitude. Synthesizing arbitrary optical waveforms first requires broadband coherent light. Most lasers are narrowband: for example, a laser pointer may emit only in red or green wavelengths. Thus, a large number of laser beams emitting over infrared, visible, and ultraviolet spectral regions would be needed as a source. The required range of frequencies to cover the optical spectrum (the bandwidth) approaches 1 PHz.

Light with different frequencies (colors) can be combined to synthesize optical waveforms such as square and sawtooth waves, as well as short pulses.

A second requirement is precise control over the phase and amplitudes of the frequencies (called spectral components) to allow for temporal shaping of the output (creating pulses of longer or shorter duration). As illustrated in the figure (see the figure), each beam of a particular color is a sinusoidal wave with a certain frequency, and appropriate superpositions of these waves result in arbitrary waveforms. Different waveforms are obtained by varying the phase and amplitude of the different frequencies.

At the heart of the approach of Chan *et al.* is molecular modulation (2–7). This technique, pioneered by Harris's group at Stanford University, uses the excitation of vibra-

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tional and rotational transitions of molecules to modulate laser light at very high frequencies. Using the fundamental vibrational transition in molecular hydrogen, Chan *et al.* generated five spectral components ranging in wavelength from 2406 nm (infrared) to 481 nm (orange). The frequency difference between adjacent spectral components is 125 terahertz (THz), which is the vibrational frequency of hydrogen. By adjusting the phase and amplitude of each spectral component, they synthesized trains of square, sawtooth, and other waveform pulses (typically with a width of a few femtoseconds). Although the current experiment uses only five spectral components, future improvements on the technique may result in the generation of thousands of spectral components covering the full optical region of the spectrum.

If perfected, an arbitrary optical waveform generator has broad implications for a number of research areas. If the spectral components are all brought into phase, flashes of light that last only 0.1 fs are formed. These ultrashort pulses of light are ideal for probing processes in atoms, molecules, and solids that occur at the subfemtosecond time scale. Although 0.1-fs-long

pulses are routinely produced in the soft-x-ray region through high harmonic generation (8–10), synthesizing such pulses in the more accessible optical region will expand the scope of ultrafast imaging.

Another application is in quantum control, in which light pulses are used to control the excitation of states in atoms and molecules (11, 12). Efficient quantum control can be achieved only if the spectral components of the light match all possible excitations in the system. By using arbitrary optical waveforms with a broad spectrum, it should be possible to control electronic, vibrational, and rotational coordinates at the same time and induce chemical reactions.

Recently, there has been some progress in extending the molecular modulation technique from excitation with short laser pulses to excitation with continuous-wave (CW) laser light (13, 14). These efforts may produce a broad spectrum in which each spectral component is a narrow-linewidth CW beam. Precision spectroscopy of atoms and molecules would be possible by choosing components of the spectrum that are close to spectral transitions of interest. Furthermore, the whole spectrum can be locked to a frequency refer-

ence so that the absolute frequency of each spectral component is known to a very high precision. With this approach, it may be possible to simultaneously produce thousands of optical clocks covering the full optical region of the spectrum (15, 16).

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BIOCHEMISTRY

Molecular Motors, Beauty in Complexity

James A. Spudich

Among the most fascinating enzymes are the molecular motors, which exquisitely couple adenosine triphosphate (ATP) hydrolysis to directional mechanical motion. They power the movement of intracellular vesicles, chromosomes, and messenger RNA–protein complexes through the cytoplasm of nearly all eukaryotic cells, using actin filaments and microtubules as their tracks. A prominent theme in these motors is allostery, or communications that occur across the enzyme at several nanometer distances. Chemical events occurring in the motor's active site, for instance, are coordinated with tight binding of the motor to the track along which it moves, and then its subsequent release, and with mechanical elements that amplify small movements occurring near the active site. In the 1980s,

researchers used quantitative in vitro motility assays, sensitive to single molecules, to study two of the three major classes of motor enzymes: the microtubule-based kinesin family and the actin-based myosin family. In the 1990s, investigators solved the crystal structures of kinesin 1 (1) and muscle myosin II (2). These complementary approaches ushered in a new era of understanding the mechanisms of these molecular machines. Dynein, the third important class of molecular motor, is a complex that processively moves along microtubules in the opposite direction to kinesin 1. Although single molecule assays have been applied to dynein, detailed structural information on this mammoth machine has remained elusive, until now. On page 1159 of this issue, Carter *et al.* (3) report a crystal structure for a 610-kD homodimer of yeast cytoplasmic dynein. The structure reveals surprises about how this massive molecular motor might work.

X-ray crystallography provides some surprising insights into the dynein class of molecular motors.

Dynein was first discovered by Gibbons in 1965 (4) as the adenosine triphosphatase (ATPase) that drives the beating of cilia and flagella. Subsequent studies showed that dyneins play diverse motility roles in eukaryotic cells. Paschal *et al.* (5) identified a cytoplasmic version of dynein, which subsequent studies showed powers the movement of many cargoes. A final type of dynein (cytoplasmic dynein 2) powers the movement of protein building blocks in cilia and flagella.

Given how much we know about kinesins and myosins from structural and single-molecule studies, one might think that we could make a reasonable guess about how dynein works. However, dynein emerged from an evolutionary lineage that is separate from kinesin and myosin (which share an ancient evolutionary origin) and seems to be a completely different type of molecular machine. Phylogenetic sequence analysis (6) showed that dynein is a member of the AAA fam-

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ily (ATPases associated with diverse cellular activities), but it is an odd uncle. Many AAA ATPases are protein unfoldases, which target proteins for degradation or break apart protein complexes (7). These AAA ATPases work by self-assembling into hexameric rings and then use ATP energy to “stuff” the polypeptide chain into the central pore. Some AAA proteins also act as DNA and RNA helicases, again by feeding the nucleic acid polymer through the central pore. Dynein, however, has evolved a way of using the same basic ATPase module to walk along a microtubule track.

Dynein also is more complicated than most AAA ATPases. It has integrated six AAA domains (four are functional for ATP binding and two are nonfunctional) into one polypeptide chain that folds into a ringlike structure (see the figure). Dynein also has added various bells and whistles to this basic ring-shaped scaffold, which allow it to function as a cytoskeletal motor. One such additional feature is a mechanical element, called the linker, which sits on top of the ring and might swing from one position to another, perhaps similar to the lever arm of myosins (8). A second feature is a long (~15 nm) coiled coil, called the stalk, which emerges from the ring and has a microtubule binding domain perched on its end. Remarkably, the affinity of the microtubule binding domain is modulated by transitions in the ATPase cycle (the binding of ATP, hydrolysis and product release), primarily by one of the four AAA ATPase domains (AAA1). However, this ATPase site resides on the opposite side of the ring from where the stalk and microtubule binding domain protrude out of the ring. How an allosteric conformational change propagates from AAA1, around the ring, and then through a long coiled coil to the microtubule binding domain remains a mystery.

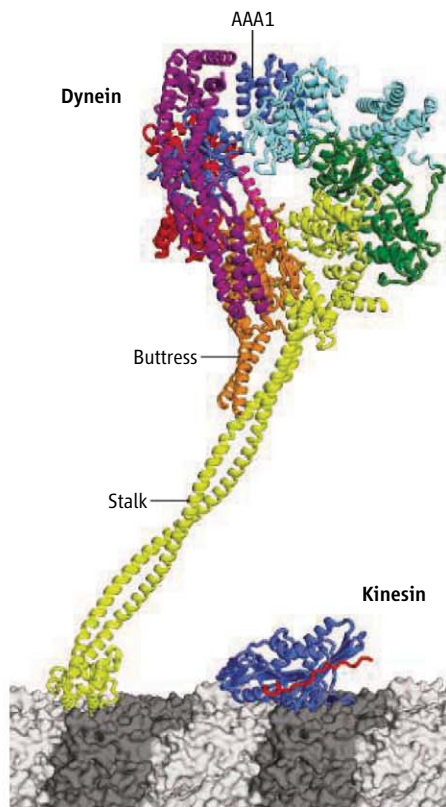
Past studies using EM were instrumental in providing the first structural insights into dynein, revealing the approximate locations of the AAA domains, the linker, and the stalk, and showing the movement of the linker in different nucleotide states (9, 10). Carter *et al.* have taken the problem an important step further with x-ray crystallography, solving a 6 Å structure of the cytoplasmic dynein motor domain. The structure is not of high enough resolution to resolve the amino acid side chains, but it shows virtually all of the helices and β sheets. For a complex machine like dynein, however, such information is important because it reveals the organization and interactions of the ATPase domains, how the stalk integrates into the ATPase ring, and the secondary structure of the linker.

The structure uncovered several surprises. First, the microtubule binding stalk appears to be supported by a second coiled coil that emerges from the ring. The authors call this second and shorter coiled coil the “buttress.” However, it may not just prop up the stalk; it may also play an important role in changing the structure of the stalk’s coiled coil during dynein’s ATPase cycle in a way that alters microtubule binding affinity. Second, the linker, which is composed of helical bundles, does not sit flat on the ring but rather arches over it, looking a bit like the handle of a basket. The contact of the linker with one end of the ring looks tenuous, however, suggesting that it may break and come loose at some stage of dynein’s ATPase cycle. Third, the ATPase domains do not form a symmetric hexameric ring, as predicted (11), but instead are arranged in a very irregular pattern. Partic-

ularly intriguing is a large gap between AAA1 (dynein’s main hydrolytic site) and AAA2. This is surprising, because hydrolysis of ATP requires a neighboring subunit in close proximity to provide key residues that promote γ -phosphate bond cleavage. The authors crystallized dynein without nucleotide and speculate that if ATP bound to AAA1, it would draw AAA2 closer, thus decreasing the gap and allowing hydrolysis to occur. The movement of AAA2 toward AAA1 might start a domino effect of movements of other AAA domains around the ring. Such a domino effect could explain how binding of ATP to AAA1 could affect the stalk/microtubule binding domain, the buttress, and the linker, even though they are on the opposite side of the ring.

Many questions remain unanswered. Carter *et al.* do not address the role of nucleotide in other AAA domains (AAA2, AAA3, and AAA4), and it is unclear at this resolution whether these sites might contain tightly bound nucleotide. We also are left with the question of why it was advantageous for cells to have evolved such a mammoth motor, when a much smaller microtubule motor, kinesin, is available. Perhaps the answer lies in the specific mechanisms that regulate dynein motor function. Co-crystals of the dynein motor domain with some of its regulatory proteins (e.g., lissencephaly 1) might provide answers.

Finally, this is only one snapshot of the motor in action. Obtaining views in two or more nucleotide states is essential. To ultimately understand how dynein works, researchers will need crystal structures of dynein with better than 3 Å resolution, and in different nucleotide states. This first structure, however, has provided a wealth of information, new hypotheses that can be tested, and optimism that crystals of different nucleotide states may be obtained in the near future.



Structure of the dynein motor. Dynein is depicted bound to a microtubule next to the motor domain of kinesin 1. The six AAA domains (dark blue, light blue, green, yellow, orange, and red), linker (purple), buttress, and stalk are indicated. The affinity of the microtubule binding domain is modulated by transitions in the ATPase cycle, primarily by the AAA1 ATPase domain (dark blue). In Carter *et al.*’s crystal structure, the stalk was truncated just below the point at which the buttress meets the stalk. The structure of the distal microtubule binding domain is from (12), and an intervening coiled coil of the proper length is introduced in this figure.

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CANCER

New Epigenetic Drivers of Cancers

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Exome sequencing—the targeted sequencing of all protein-coding regions in the human genome—now offers an unprecedented opportunity for systematic, genome-wide discovery of somatic mutations in tumor tissue. On page 1199 of this issue, Jiao *et al.* (1) have applied this pioneering technique to identify common genetic mutations underlying pancreatic neuroendocrine tumors (PanNETs), a class of tumors that frequently arise from pancreatic islet cells. This report adds to a growing number of studies that use exome sequencing to explore cancers with unknown or poorly understood genetic etiology (2–8).

The diagnosis and treatment of nonfamilial PanNETs present clinical challenges, and little is known about the genetic mutations in these tumors. In addition to the well-studied tumor suppressor genes *PTEN* and *TSC2*, which antagonize the mammalian target of rapamycin (mTOR) growth signaling pathway, Jiao *et al.* found that more than 60% of PanNETs are mutated in at least one of three genes involved in modifying chromatin (see the figure). From the 68 patients

examined, 30 carried mutations in *MEN1*, 17 in *DAXX*, and 12 in *ATRX*. Whereas simultaneous mutations of *ATRX* and *DAXX* were never observed in the same tumor, lesions in either *ATRX* or *DAXX* coincided with *MEN1* mutation in 16 cases. Interestingly, mutations in these three genes were associated with prolonged patient survival when compared to mutations in genes encoding classical tumor suppressors.

In the past decade, chemical modification of DNA or histones (which together form the nucleosome units of chromatin), as well as noncoding RNAs, have been recognized to play critical roles in animal development as regulators of gene expression and carriers of epigenetic information. Chromatin structure and its remodeling into states that are accessible and inaccessible to gene transcription are thought to play vital roles in preventing genomic instability and tumorigenesis. Nonessential, tissue-specific genes and genes required for pluripotency are repressed in differentiated cells. Through the establishment and maintenance of specialized chromatin signatures that render these genes inaccessible, epigenetic pathways help to maintain cellular identity. Mutations in epigenomic regulators have the potential to alter these states, leading to misregulation of gene expression

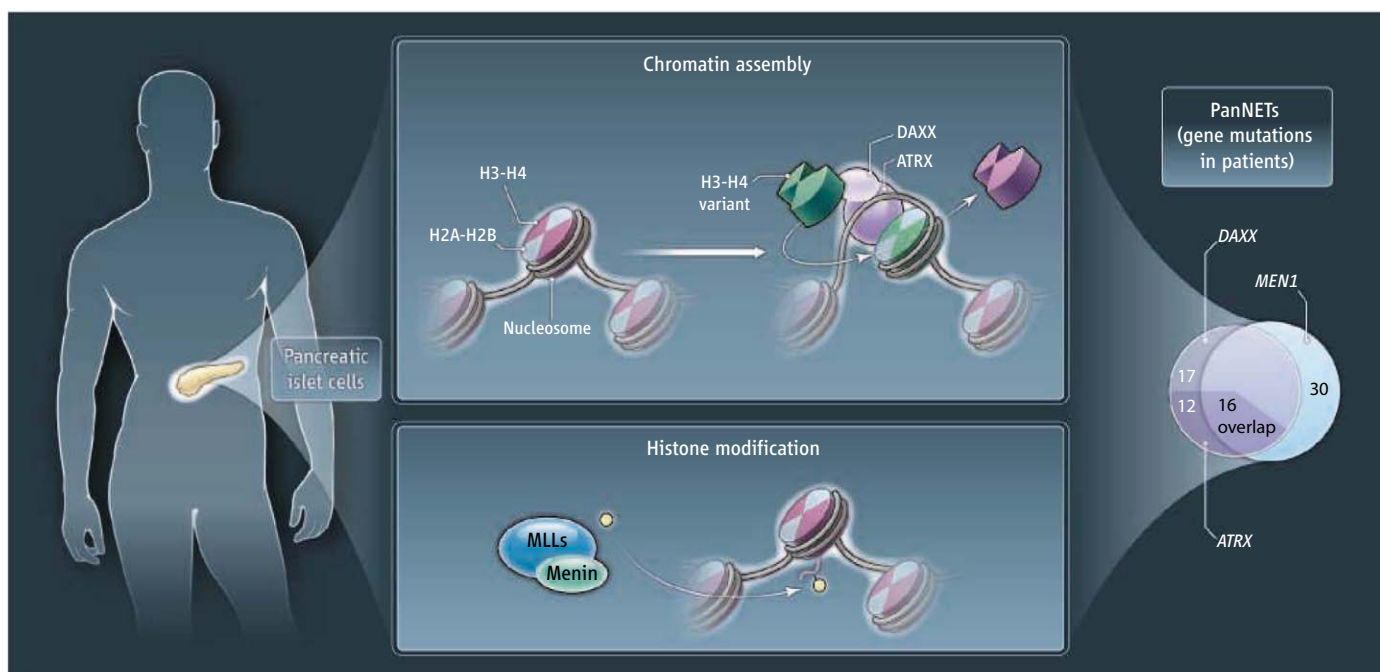
Mutations in proteins that control chromatin dynamics contribute to tumorigenesis.

that contributes to tumorigenesis.

The identification of both *ATRX* and *DAXX* mutations in PanNETs was particularly surprising. *ATRX* is a SNF-2 family chromatin-remodeling adenosine triphosphatase that has been extensively studied in the context of the α thalassemia–mental retardation X-linked syndrome (9). Males affected by this disorder suffer from a variety of developmental defects during early childhood. At present, more than 100 distinct germline mutations in *ATRX* have been described that affect the encoded protein but are thought to retain partial activity. By contrast, Jiao *et al.* find frameshifts and nonsense mutations that cause a complete loss of *ATRX* and *DAXX* protein in the majority of PanNET cases. The multifunctional protein *DAXX* associates with *ATRX*, and this complex has been linked to deposition of histone H3 family member H3.3 at heterochromatic (transcriptionally silent) regions of the genome, including the ends of chromosomes (telomeres), but also at tan-

Chromatin regulators and tumors. Epigenetic regulators Menin and ATRX-DAXX may promote genome integrity and maintain cell identity through the modification of chromatin structure. Loss of these pathways plays a role in the development of PanNETs.

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dem repeats enriched in guanine near active genes (10–13).

Features intrinsic to repetitive elements—such as DNA bending and the propensity to adopt so-called G-quadruplex structures—can adversely affect nucleosome occupancy, and the ATRX-DAXX complex might re-establish H3.3-containing nucleosomes at these elements in a replication-independent manner. Defective nucleosome assembly pathways likely lead to increased DNA damage and genome instability. At telomeres, the ATRX-DAXX complex is required to suppress aberrant DNA repair that can result in telomere fusion (14). Furthermore, defects in chromosome congression, cohesion, and centromere function caused by the loss of ATRX may promote missegregation of chromosomes and consequential aneuploidy (15). A recent study found that 98% of PanNETs exhibit substantial chromosomal alterations (16).

Originally associated with familial endocrine tumors, the *MEN1* gene is the most frequent genetic lesion in both heritable and sporadic neuroendocrine tumors, including 43% of the PanNETs in the study by Jiao *et al.* *MEN1* encodes the transcription factor Menin, which recruits the H3K4me3 histone methyltransferase mixed-lineage leukemia (MLL) complex. *MLL2* and *MLL3*, which encode methyltransferase components of MLL complexes, are frequently mutated

in childhood medulloblastomas (5). Given the prevalence of *ATRX*, *DAXX*, and *MEN1* mutations in PanNETs, it is conceivable that inactivation of either or both complexes is required for tumorigenesis. In addition to their pleiotropic functions during development, these epigenetic regulators therefore appear to function as potent tumor suppressors in pancreatic islet cells.

Although the molecular mechanisms of their tumor suppressor activities have yet to be defined, Menin and the ATRX-DAXX complex are part of a growing list of chromatin-associated tumor suppressors. Several recent cancer exome studies have identified mutations in pathways involved in either the methylation or demethylation of H3K27, a histone modification associated with genomic silencing (3, 6, 17). Mutations in chromatin-remodeling complexes related to ATRX-DAXX were identified in a variety of cancers. Nearly half of all clear cell ovarian tumors contain mutations in the *ARID1A* gene, encoding the BAF250 subunit of the human SWI-SNF chromatin-remodeling complex (4, 8). The gene encoding another human SWI-SNF subunit, BAF180, is mutated in 41% of clear cell renal cancers (7). Mutations in other human SWI-SNF subunits have previously been identified in a variety of specific cancers.

Much work remains to define the role of these and other epigenetic regulators in different tumor types. A major unresolved ques-

tion is how mutations in different subunits of multiprotein complexes lead to disparate types of cancers. The occurrence of these mutations in defined subsets of tumors suggests that epigenetic factors may act in a tissue-specific manner to suppress oncogenic pathways upstream of master regulators common to a broader range of tumors. Identification of this diverse set of “backseat drivers” through technological advances in genome-wide analysis will provide spectacular opportunities for advanced diagnostics and treatments for a wide variety of human cancers.

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MATERIALS SCIENCE

Local Peeling of Graphene

Daniel Gunlycke and Paul E. Sheehan

“Perfection is finally attained not when there is no longer anything to add, but when there is no longer anything to take away,” noted Antoine de Saint-Exupéry (1). He could have been writing about graphene sheets, just an atomic layer or two thick, which have properties much more interesting than those of the bulk. From the time when graphite would be rubbed on an insulating surface with the hope that one of the exfoliated flakes would be a single layer, graphene manufacture has progressed rapidly and is now routinely grown on or transferred onto many substrates, even up to sizes large enough for TV displays (2). Despite such advances, reproducible spatial

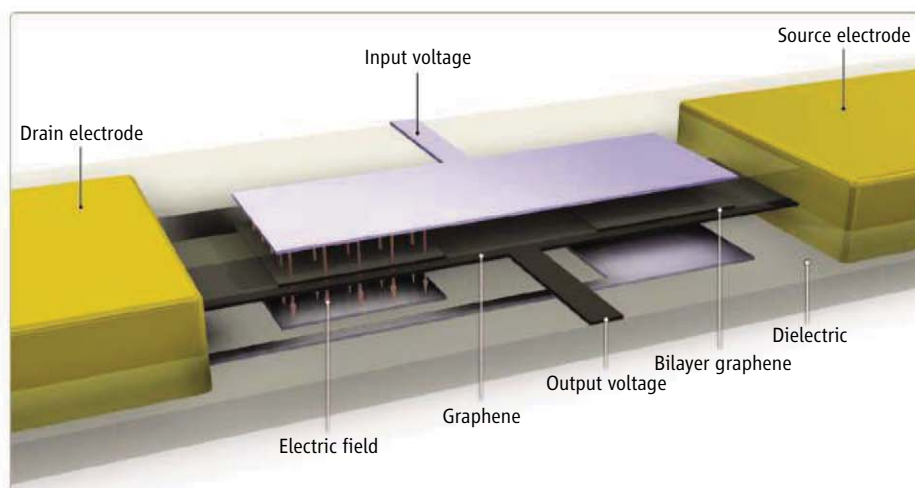
control over the number of graphene layers has not been achieved. On page 1168 of this issue, Dimiev *et al.* describe a technique that overcomes this limitation and allows peeling of graphene layer by layer at predetermined locations of the surface (3).

Achieving such control is important because electrons in a double layer of graphene (a bilayer) behave quite differently from those in a single layer (a monolayer). A monolayer of graphene is a semimetal with no band gap and conical-shaped conduction and valence bands close to the Fermi level. Although the conical dispersion leads to exceptional electronic transport properties, the lack of a band gap limits monolayer graphene’s use in conventional electronics—without a band gap conduction through the device cannot be switched on and off. One

A technique is demonstrated that allows single layers of graphene to be removed one layer at a time.

route to a band gap is to cut graphene into nanoribbons so that the electrons are laterally confined. Although nanoribbons have been formed by several groups, the routine fabrication of ribbons with widths of 2 to 50 nanometers is challenging, as rough edges scatter electrons, thereby masking the desired electronic properties (4). A different route to a band gap is using bilayer graphene that has the same stacking (Bernal) as natural graphite. In this particular stacking configuration, the additional layer results in a more typical parabolic dispersion but, more importantly, produces a controllable band gap in the presence of an electric field normal to the plane (5). Devices that capitalize on both the conical dispersion of monolayer graphene and the controllable band gap of bilayer graphene should be especially powerful.

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The technique of Dimiev *et al.* provides reproducible control over the number of graphene layers spatially on a surface. They first sputter thin films of zinc metal onto selected areas of few layer graphene. Exposing the zinc-coated sample to a mild aqueous solution of HCl removes only the areas in contact with the zinc, leaving both the uncoated and the underlying layers intact. Additional experiments suggest that the process works when the metal (i) damages the graphene during deposition, (ii) has a large oxidizing potential, and (iii) reacts with the etching solution to form hydrogen gas. The process is surprisingly robust, capable of peeling layers from several different varieties of graphene, including graphene oxide. Notably, they demonstrate that the process may be repeated to generate multiple patterned layers. Thicker films of graphene may be selectively thinned and patterned to generate the final device.

There is great potential for combining different thicknesses of graphene in a device structure such as a complementary metal-oxide semiconductor inverter, a cornerstone of digital electronics (see the figure). Such a device could be fabricated by depositing a Bernal-stacked bilayer of graphene onto existing electrodes and then peeling some areas away to form monolayers. The bilayer graphene regions in the center are transistors that ideally conduct only in the absence of a vertical electric field. Vertical electric fields in the bilayer regions are controlled by gate electrodes in the top and bottom layers of the device that are electrically insulated from the central layer. Monolayer graphene, which remains an excellent conductor in the presence of these fields, is an ideal lead material to the source and drain electrodes. Note that this device could be truly “all carbon” by making both the top and bottom gates from graphene. Moreover, insulating forms of graphene gen-

erated through fluorination (6) could serve as thin and effective gate dielectrics. Clearly, several practical challenges (for example, small band gap and cleanliness) remain; however, this device does illustrate one of many new structures more readily achieved with this new fabrication technique.

The exact mechanism for graphene peeling remains unclear, so other metals or materials might also be effective. The details of graphene’s reaction with energetic metal ions would be particularly interesting because in the current process only a few of the deposited metal atoms have sufficient energy to

A peeling potential. An idealized graphene device that would take advantage of local layer-by-layer peeling (3). The central black layer contains regions of monolayer and bilayer graphene and is insulated with a dielectric from the top and bottom gate electrodes. Application of an input voltage to the top gate that matches that of the drain (or source) electrode would generate an electric field in the left (or right) bilayer graphene region. The electric field suppresses conduction, leading to an output voltage matching that of the source (or drain) electrode, effectively inverting the input voltage.

modify the graphene. Moreover, the lateral resolution of the technique is ripe for exploration, given the desire for ever smaller electronic devices. Ultimately, the ability to peel just a single layer of graphene from a desired area with such a simple and robust technique is exceedingly useful. Local graphene peeling should become a routine tool for researchers to explore new devices.

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MICROBIOLOGY

Establishing the Secretion Hierarchy

Luisa M. Stamm and Marcia B. Goldberg

A large complex of type III protein secretion system components helps bacteria to invade their hosts.

Type III protein secretion systems (T3SSs) are specialized complexes of molecules in Gram-negative bacteria that mediate the introduction of virulence proteins directly into eukaryotic host cells. These supramolecular structures span the bacterial membranes, cross the extracellular space, and penetrate host cell membranes. The proper function of these systems depends on sequential secretion of the needle components, the proteins that enable

translocation across the host cell membrane (translocases), and the effector proteins that carry out virulence functions (1). How this hierarchical process is regulated, however, has been unclear. On page 1188 of this issue, Lara-Tejero *et al.* (2) demonstrate that a large complex of T3SS proteins in *Salmonella enterica* serovar Typhimurium serves to sort components of the system for secretion in the appropriate sequence. Also, on page 1192, Schraidt and Marlovits (3) define the precise symmetry and stoichiometry of proteins that constitute the base of the needle complex.

T3SSs are present in many bacterial pathogens, including *Salmonella* spp., *Shi-*

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gella spp., *Yersinia* spp., pathogenic *Escherichia coli*, and *Pseudomonas* spp. (4). T3SSs secrete several hundred effectors that manipulate the host cell and enhance bacterial survival and dissemination. Effectors vary in structure and display a range of functions, including dampening the immune response, inducing or preventing bacterial uptake, and controlling the host's cell cycle and death. In contrast, the secretion apparatus of T3SSs consists of approximately 20 proteins that are highly conserved across species.

The assembly of the needle complex portion of the secretion apparatus begins with insertion into the bacterial membranes of three proteins (PrgH, PrgK, and InvG in *Salmonella* spp.). These proteins oligomerize and interact to establish a membrane- and peptidoglycan-spanning channel, designated the needle complex base, which provides the structural framework for the T3SS. In their high-resolution structure, Schraidt and Marlovits show elegant 24:24:15 symmetry of PrgH:PrgK:InvG. Once the needle complex base is assembled, the early T3SS substrates that constitute the needle filament and inner rod (PrgI and PrgJ) are secreted (see the figure). In *Salmonella* spp., needle length is controlled by the protein InvJ; its absence leads to the formation of long needles (5). When the needle is complete, the needle tip protein (SipD) is secreted, at which time the apparatus is ready for use. After activation of the system by contact with host cells, two translocases (SipB and SipC) are secreted through the apparatus and form a pore in the host cell membrane (6). The system then undergoes a switch and begins transferring effectors into the host cell. The tight temporal regulation of this sequence results in the translocation of effectors only when the assembled apparatus is attached to host cells and the continuous conduit from the bacterial cytoplasm to the host cytosol is open.

Lara-Tejero *et al.* provide new insights into the mechanism that establishes this temporal hierarchy by identifying SpaO as a key player in controlling sequential secretion of proteins through the *Salmonella* T3SS. Previously, little was known about SpaO, other than that it is required for T3SS secretion and invasion of host cells (7) and that homologs in other organisms are present in a high-molecular weight complex (8, 9). These investigators now find SpaO in such a high-molecular weight complex that includes the needle complex, the translocases, and OrgA and OrgB. These findings suggest that the translocases might be docked on the SpaO-OrgA-OrgB complex, "poised" for secretion upon host cell contact. Indeed, in the absence of the



Model for queuing T3SS substrates in *Salmonella*. (A) Secretion of the needle components PrgI and PrgJ with elongation of the needle. InvJ controls needle length. (B) Completed assembly of apparatus: SipD at the needle tip and translocases SipB and SipC and their chaperones docked on the sorting complex, poised for secretion. (C) Upon activation, translocases are secreted and inserted into the host membrane, and effector-chaperone complexes associate with the sorting complex for secretion.

translocases, the SpaO-OrgA-OrgB complex associates with T3SS effectors. The investigators postulate that if the SpaO-OrgA-OrgB complex functions in sorting T3SS substrates, then needle components, but not translocases, should be docked during active assembly of the needle. As predicted, in a mutant strain lacking the InvJ protein, which constitutively secretes the needle filament protein (5), the SpaO-OrgA-OrgB complex lacks SipB. Together, these data support the hypothesis that the SpaO-OrgA-OrgB complex serves as a platform for "sorting" proteins to be secreted through the T3SS in a hierarchical fashion. The observation that *Shigella* spp. homologs of SpaO, OrgA, and OrgB also form a complex that recruits effectors for secretion (3, 8, 9) suggests that sorting platforms will be conserved across T3SSs.

Although these findings establish the existence of a sorting platform that regulates the ordered secretion of substrates, they also raise questions about the molecular mechanisms that mediate this process. In the bacterial cytoplasm, translocases and certain effectors are bound by chaperones; for some, proper sorting on the SpaO-OrgA-OrgB platform is dependent on association of the secreted protein with its chaperone.

It remains unclear which aspect of the substrate-chaperone complex provides hierarchical information, and how recognition occurs for effectors that lack chaperones. Perhaps, as Lara-Tejero *et al.* suggest, the substrates and/or their chaperones associate with the sorting complex according to affinity, such that those substrates with the highest affinity are sorted and secreted earlier. Future studies of the SpaO-OrgA-OrgB complex will further elucidate, at a molecular level, the hierarchical control of T3SSs; such studies might identify new therapeutic targets that could be broadly applicable to Gram-negative pathogens that express these systems.

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Controlling and Coordinating Development in Vector-Transmitted Parasites

Keith R. Matthews

Vector-borne parasites cause major human diseases of the developing world, including malaria, human African trypanosomiasis, Chagas disease, leishmaniasis, filariasis, and schistosomiasis. Although the life cycles of these parasites were defined over 100 years ago, the strategies they use to optimize their successful transmission are only now being understood in molecular terms. Parasites are now known to monitor their environment in both their host and vector and in response to other parasites. This allows them to adapt their developmental cycles and to counteract any unfavorable conditions they encounter. Here, I review the interactions that parasites engage in with their hosts and vectors to maximize their survival and spread.

Our understanding of the important steps in a parasite's life cycle is usually dominated by where they cause disease. This is inevitably their human and animal hosts, where the study of the strategies that parasites use to ensure survival and proliferation have shaped our thinking of immune system function, as well as of eukaryotic cell and molecular biology. From the parasite's perspective, in fact, this might not be the most challenging part of their life history, because overcoming the immune system leaves them in a nutritious and equitable environment. Less predictable challenges come when parasites leave their host, aiming to colonize new hosts. Although some parasites achieve this, for example, by releasing resistant dormant stages that are directly transferred to new hosts in food or water, many parasites rely on vectors for their transmission (Fig. 1). However, parasites do not simply evade host immunity until a passing vector picks them up but instead use exquisitely controlled mechanisms of environmental sensing and developmental regulation to ensure their transmission. This imperative has dominated the evolution of many parasites to the extent that the drive for transmission influences almost every aspect of their biology, including interactions within their vector, often a blood-feeding arthropod. Here, I review our state of understanding of how parasites optimize their transmission potential and overcome the challenges they encounter as they passage through their vectors. Initially, I address how parasites prepare for their uptake by a vector, by optimizing production of specialized transmissible forms, which often exhibit arrested development and sensitivity to the environmental cues that signal passage to the vector. Next, I address how parasites detect their exit from the mammalian host and rapidly adapt to enable survival and proliferation in their invertebrate vector. Lastly, I discuss how vectors combat their in-

vasion by parasites and how parasites have evolved strategies to manipulate their vector to maximize their transmission.

Preparing for Transmission: How Do Parasites Maximize Their Chances of Success?

Once parasites become established in a mammalian host, they must prolong their survival and maximize their likelihood of transmission to a new host. These might be complementary objectives, but often they require a trade-off that is managed by cell-type differentiation to generate developmental forms specialized for each role. Simplistically, a consistent proportion of each cell type might be sufficient to achieve transmission; however, the host is a dynamic environment, where variable exposure to immune responses, competing parasite strains, and the stresses associated with infection or drug exposure can generate opposing pressures on survival and transmission. Consequently, parasites monitor their environment and respond by altering their investment in different developmental forms. For example, in the malaria parasite *Plasmodium*, asexual stages predominate to ensure that the population of infected red blood cells is maximized. Subsequently, the development of sexual stage gametocytes prepares the parasite for transmission (1). However, developmental investment shows plasticity under different host conditions, such that the proportion (2) and even the sex ratio (3) of transmissible gametocytes can change in response to stress or competition. This interaction with the host-modulated environment is also seen in some parasitic helminths, where the hormonal context and exposure to CD4⁺ cells drive schistosome blood-fluke development (4), and in filarial nematodes, where experimental exposure to a more aggressive immune environment promotes the production of transmissible microfilaria (5).

Parasites also interact with related parasites to maximize their probability of transmission. For example, *Trypanosoma brucei*, which causes human African trypanosomiasis controls its investment in transmissible "stumpy" forms by monitoring its cell density in the host bloodstream

(6), a phenomenon akin to bacterial quorum sensing. It does this apparently by releasing a signal, termed stumpy induction factor (SIF). SIF has so far eluded purification perhaps because it represents a complex mixture of small molecules or because the bioassays for its detection have been insufficiently robust. Nonetheless, the potential importance of this unidentified factor is clear because a variability between SIF production and turnover in different hosts could influence the number of transmissible parasites at a given cell density. Because African trypanosomes infect a wide range of mammals, which also act as reservoirs for human infection, this variation would affect the zoonotic potential of the parasite. Moreover, if cell density signaling is effective between *T. brucei* strains, as appears to be the case from one laboratory study (6), then a conflict between parasites in a co-infection could be established. This conflict has the potential to select different strains that differ in their production or sensitivity to SIF or that are optimized to different hosts, thus affecting their probability of transmission in different contexts.

As well as their ability to sense and respond to their local environment, a further characteristic feature of transmission stages for both metazoan and protozoan parasites is the capacity to arrest their development. Frequently, parasites stop proliferation before undergoing the differentiation responses that generate transmissible forms. This strategy is similar to that seen in other eukaryotic cells that become quiescent in order to tolerate harsh environmental changes. Developmental arrest has the obvious advantage of limiting the risk of lethal cell division errors that can occur during DNA replication or chromosome segregation. Crucially, for parasites, it can also prevent uncontrolled proliferation in the host, which if unchecked could lead to host damage or death and so limit transmission potential.

Unlike many quiescent eukaryotic cells, the arrested development of parasite transmission stages is usually irreversible. A consequence is that transmission stages must be stringently regulated to prevent premature differentiation in the mammalian host, a response that would be lethal to the parasite. In African trypanosomes, such forward development is tightly repressed by a tyrosine phosphatase, which holds the parasites poised for differentiation (7). The arrested transmission stages must also be able to persist long enough to favor their uptake by a vector to continue the life cycle. Several mechanisms achieve this in different parasite groups. In *T. brucei*, one strategy is to use the hydrodynamic flow generated by parasite swimming to sweep surface-bound antibody toward the parasite posterior, where it is internalized via the flagellar pocket (8). Generalized immunosuppression is another common strategy used by parasites to potentially prolong the longevity of transmission stages. For example, in filarial infections serpins specifically secreted by transmissible microfilariae are able to modulate host immune function (9).

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Clearly, parasites optimize their probability of transmission by interacting with the network of signals operating between the host, kin, and competing parasites (Fig. 2). We assume complex sensing mechanisms have evolved, but we know little about them; for instance, how is *Plasmodium* gametocytogenesis initiated or modulated? What is SIF? How are competitors or immune factors recognized and responded to in different parasite groups? Despite functional investigations on individual signaling components that affect transmissibility and the complete cataloging of conventional signaling molecules identified in genome analyses, there is almost no coherent understanding of the molecular pathways that regulate parasite development. An essential current goal, therefore, is to assemble these pathways. Understanding the molecules and mechanisms that control the interactions between parasites and hosts, and among parasites themselves, also offers the prospect of approaches to disease control that manipulate the parasite's transmission potential.

Once Transmitted, How Do Parasites Rapidly Adapt to Their Vector?

The uptake of parasites by their vector requires that they perceive environmental change and adapt immediately to the new host. Unsurprisingly, temperature change is a major determinant of environmental sensing when moving from a mammalian host to an invertebrate vector (10). However, temperature alone is not sufficient, and at least two signals are usually required to precipitate differentiation, perhaps providing a failsafe to prevent the risk of premature development in the mammalian host. For *Plasmodium*, a component of the mosquito eye-pigment synthetic pathway, xanthurenic acid, is a key environmental trigger (11) when sensed in the context of both low temperature and raised pH. Temperature is also an important cue for *Leishmania* spp. and *Trypanosoma cruzi* during their development, whereas for African trypanosomes, low temperature enables the detection of citrate in the blood meal, which then triggers differentiation (12). Once the stimulus is perceived, signaling cascades are activated, mediated through cyclic guanosine 3',5'-monophosphate in *Plasmodium* (13) or a phosphatase cascade in *T. brucei* (14), for example.

Once the adaptive mechanisms are triggered, the parasites can tolerate rapid changes in the blood meal environment and evade invertebrate immune mechanisms. For such a rapid-response capability, a common strategy is to hold pre-made mRNAs ready for translation, rather than relying on new gene transcription and then protein synthesis. In *Plasmodium*, mRNAs are held silent and stable in gametocytes in P granules, storage compartments in the cytoplasm of eukaryotic cells that ensure that mRNAs are kept apart from the active translational machinery (15). *Plasmodium* P granules contain an RNA helicase, DOZI (16), that ensures mRNAs are stable and not translated until required, that is, upon ookinete

formation after ingestion by mosquitoes. A similar strategy also operates in American (17) and African (18) trypanosomes as protection against stress and probably in preparation for transmission. In *T. brucei*, transmissible stumpy forms are quiescent until they are taken up by tsetse flies,; whereupon translational repression is released, and, coupled with new mRNA synthesis, rapid adaptations to assist survival in the vector are effected (19). The signals governing the silencing of the translationally quiescent mRNA pool are

not well characterized and are unlikely to be simple motifs given the widespread repression of many genes at this life-cycle stage. Nonetheless, the recruitment to P granules of specific mRNAs required during transmission appears to be an important mechanism for holding mRNAs poised for action. Specificity in gene regulation is also expected for the small subset of genes that escape generalized translational repression operating in transmission stages. The identification of this subset will be particularly exciting because these

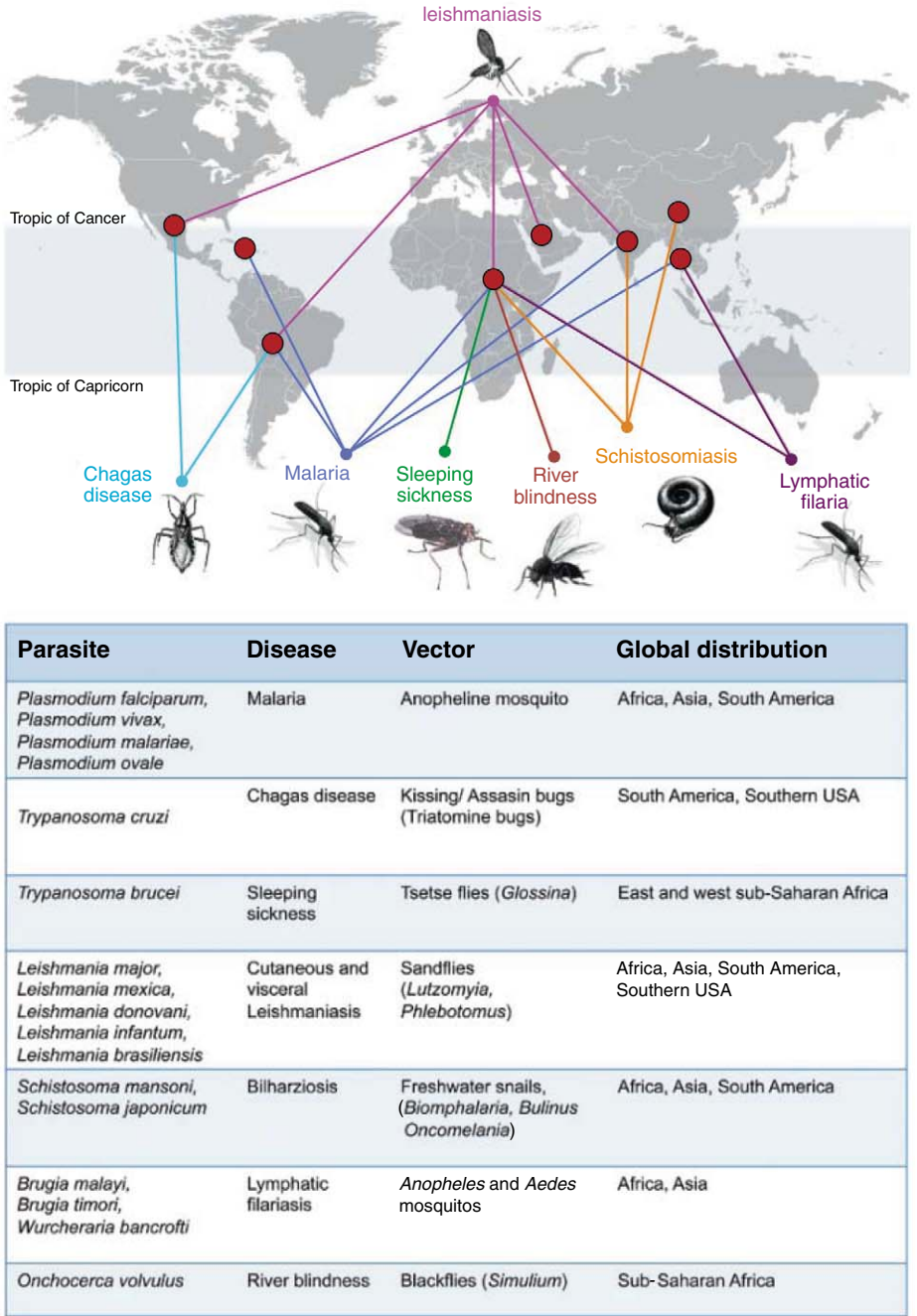


Fig. 1. The global distribution of vector-borne human diseases. The individual parasites and their vectors are indicated in the table, as are the diseases they cause and their main geographical distributions.

are the molecules most likely to be required for detecting and enacting the earliest events of differentiation in the vector. Characterization of their regulatory control is also necessary to understand the mechanisms by which parasites prepare for transmission.

In addition to protein synthesis, protein turnover is important for restructuring parasites as they adapt to their vector or prepare for transfer back to a mammalian host. In *Leishmania* parasites, an arsenal of protein degradation enzymes concentrated in the lysosome contributes to cellular remodeling. The lysosome is also the focus of autophagic events that turn over cellular proteins and organelles as the parasites alter gene expression and metabolic capacity. A similar phenomenon occurs in *T. brucei*, where the lysosome expands massively during establishment of the procyclic forms in the tsetse midgut. The expansion contributes to the replacement of stage-specific metabolic enzymes contained within the glycosomes (20), which are peroxisome-like organelles that compartmentalize the parasite's glycolytic enzymes and which show different compositions in different life-cycle stages. This replacement enables a rapid rewiring of the parasite's metabolism during the transition from one life-cycle stage to the next, which is essential as the parasite moves from a glucose-rich mammalian blood environment to the proline-rich environment of the insect midgut.

Parasites usually change morphology as they establish in their invertebrate vector. Hence, the *Plasmodium* ookinete is quite different from either the gametocytes or the zygote. The ovoid shape of ookinetes matches the basic apicomplexan form, which potentially assists their attachment to, and migration through, the mosquito gut wall. Similarly, the very short flagellum of the intracellular amastigote *Leishmania* rapidly extends when the parasite is taken up by a sandfly, allowing motility of the differentiated promastigote in the vector's gut. Morphological changes can also serve other functions. In *T. brucei* the epimastigote flagellum allows attachment to the salivary gland of the tsetse, whereas in *Leishmania* and *T. brucei* the flagellum may provide an important sensory function, which helps the parasite to monitor its environment during development (21).

Changes in the position of the mitochondrial genome (kinetoplast) with respect to the cell nucleus are also common in kinetoplastid parasites, including African and American trypanosomes and *Leishmania* sp., although the reasons are unclear. Because the kinetoplast is intimately

associated with the base of the flagellum, it may help to govern the overall length of the exposed flagellum and influence its sensory capacity. In *T. brucei*, kinetoplast repositioning may relate to the different organizational needs of the parasite's protein trafficking apparatus in the tsetse fly, where the delivery of the bloodstream variant surface glycoprotein coat to the cell surface is no longer an imperative. Alternatively, the kinetoplast may change position to assist the function of the closely located flagellar pocket or to meet distinct cell-cycle requirements in different life-cycle stages (22, 23). Indeed, the cell cycle of many parasites is intimately linked to their mor-

adapt to their new environment is important, not just to design interventions for disease control but also for understanding eukaryotic developmental biology and the coordination of functions in different organelles. In future, a more coherent picture of developmental events and their coordinated control should emerge to replace the piecemeal analysis of individual molecules that have been generated thus far.

Is There a Conflict Between Parasites and Their Vectors?

The immune evasion tactics of parasites in their mammalian host have been the focus of most research in the field; however, vector-borne parasites are also challenged by the immune defences of invertebrates (Fig. 3). Invertebrate responses consist of innate factors and cell-mediated immunity, both of which affect parasites as they establish and are sustained in their vectors. Because invertebrate immune responses share evolutionary origins with mammalian immune systems, parasites can encounter similar types of challenge. For example, in their snail vector, schistosomes are targeted by cytokines like macrophage inhibitory factor, which is homologous to the mammalian cytokine that acts against a broad range of parasites (24). After ingestion in blood, *Plasmodium* and filarial nematodes migrate through the insect's gut lining into the hemocoel, causing tissue damage and generating an antimicrobial response that can limit infection. In *Plasmodium*, ookinetes migrate through the midgut epithelium and are recognized by pathogen

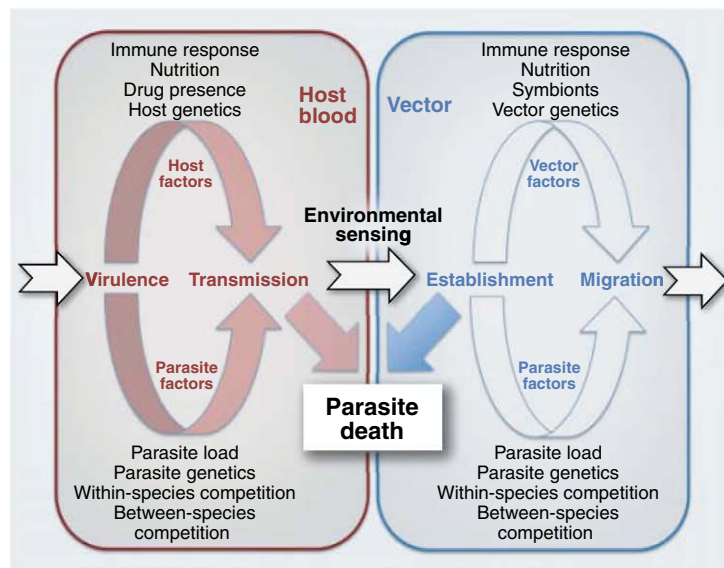


Fig. 2. Factors that influence the development of parasites as they pass through their mammalian host or vector. In each case, host- and parasite-moderated conditions can determine the developmental fate of the parasite. During transmission between the mammalian host and the vector and during establishment in the vector, there is often a significant amount of parasite death.

phology, and specific cell organization must apparently be established before embarking on cell division during differentiation in the vector.

From the analysis of the events that accompany differentiation as parasites enter their vector, it is clear that they undergo a precisely regulated and hierarchical developmental program. Many distinct cellular events have to be temporally or functionally coordinated, requiring careful control of the timing of gene expression events and the coassembly of new or modified cellular structures. How these different events are coordinated is poorly understood, despite the mapping of coincident gene expression changes and the characterization of distinct cellular events in the developmental transitions of several parasites, including *Plasmodium* and kinetoplastid parasites.

Analyzing, the extent to which different events are linked and depend on each other during the extensive remodeling that occurs as parasites

recognition receptors, thioester-containing protein 1, leucine-rich immune molecule 1, and *Anopheles* *Plasmodium*-responsive leucine-rich repeat protein 1-C, which operate to induce serine protease cascades that produce antiparasitic effects, including melanization (25), a process that targets both protozoa and helminths. Mosquitoes also express Toll and Imd receptors, which in turn stimulate antimicrobial peptide (i.e., defensins, attacins, gambicin, and cecropins) synthesis from the fat body. The immune responses generated by *Plasmodium* in concert with the community of bacteria that proliferate in a blood meal can have important consequences for parasite establishment (25). This interaction is proposed to generate a *Plasmodium*-directed immune memory in the hemocoel granulocyte population that limits subsequent transmission events by other parasite genotypes (26).

Like *Plasmodium*, procyclic-form African trypanosomes stimulate the production of an array of

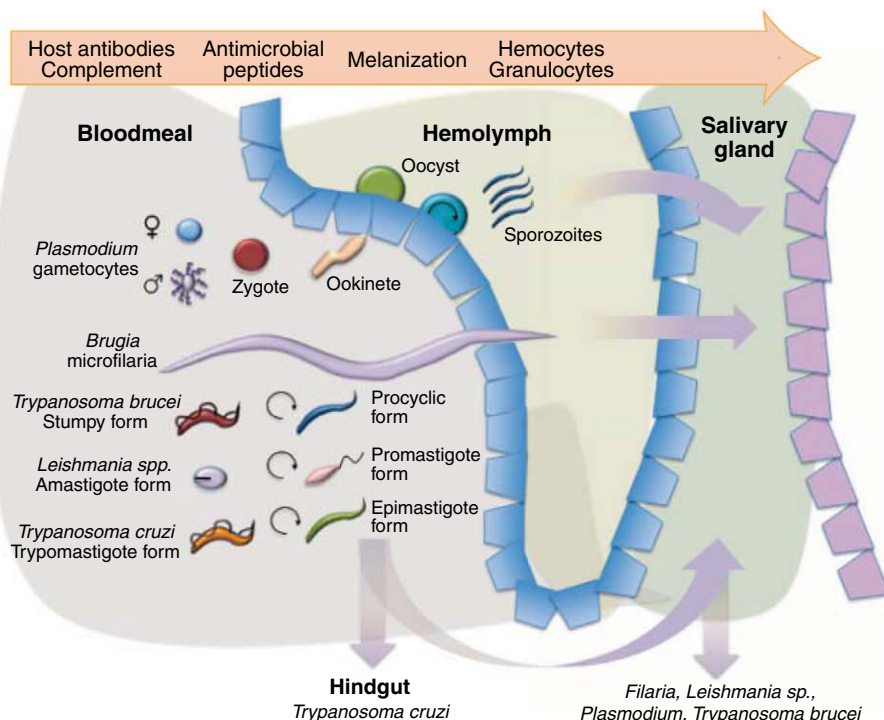


Fig. 3. Routes of transmission of different parasite groups through an arthropod vector. *Plasmodium* male and female gametocytes fuse to generate a zygote, which then matures to an ookinete that penetrates the gut lining of the mosquito vector. This develops into an oocyst, within which sporozoites develop. Sporozoites are released and migrate to the mosquito salivary gland, where they are infective to a mammalian host when the mosquito seeks a blood meal. For helminths, microfilariae are ingested during blood feeding by mosquitoes (*Wuchereria* and *Brugia*) or blackflies (*Onchocerca*). The worms then migrate through the hemocoel, eventually reaching the proboscis and salivary glands, where they can be transmitted to a new host. For kinetoplastid parasites, ingested nonproliferative transmissible forms develop into proliferative forms that establish in the alimentary canal of their vector. For *T. brucei*, the parasites then migrate via the proventriculum of the tsetse fly to the salivary glands, where they attach and multiply as epimastigote forms before forming infective metacyclic forms. For *T. cruzi* and *Leishmania*, the parasites multiply in the gut of the kissing bug and sandfly vectors, respectively, before maturing into infective forms (trypomastigote forms in *T. cruzi* or promastigote metacyclic forms in *Leishmania*) in either the hind gut or pharyngeal valve, respectively. *T. cruzi* are transmitted by expulsion during bug defecation, parasites being rubbed into the bite wound. The challenges faced by the parasite within its invertebrate vector are indicated above the diagram.

antimicrobial peptides in their vector, the tsetse fly (27), plus an unusual factor known as TsetseEP (28), composed of glutamic acid–proline (EP) repeats. This structure is similar to major surface proteins on the procyclic midgut form of the parasites known as EP procyclin, and depletion of TsetseEP promotes trypanosome establishment (29). Interestingly, this effect is not exclusively based on the sequence similarity between the two molecules, because TsetseEP also protects against *Trypanosoma congolense*, which expresses a distinct procyclin type lacking EP repeats. Reactive oxygen species are also important determinants of tsetse resistance to trypanosomes. It appears that in combination the different defense strategies are highly effective given the low frequency of trypanosome-infected tsetse in the field.

For both nematode and *Plasmodium* infections in mosquitoes, the interactions between different vectors and parasite strains in the field, compounded by the associated microbial communities, can generate complex effects on vectorial

competence (30). Here, analysis of vector immune gene sequence evolution within populations, as a signature of selection generated by parasite infection (31), offers exciting possibilities for pinpointing key molecular interactions between parasites and vectors in the field.

Although vectors mount substantial responses to limit parasite transmission, the interaction between parasites and vectors is not unidirectional. Indeed, as well as invertebrate immunity having evolved to combat parasitic invasion, parasites have evolved to manipulate their vectors to their own advantage. Perhaps the best known example is *Leishmania* parasites, which secrete a plug composed of filamentous phosphoproteoglycan in the gut of their sandfly vector. The presence of the plug stimulates the vector to increase its mammalian biting frequency and hence promotes the parasite's transmission efficiency (32). A similar phenomenon occurs during *Plasmodium* invasion of the mosquito salivary glands. Invasion of the glands by the parasite's sporozoites reduces

apyrase levels in saliva, which seems to increase the mosquito biting frequency and hence the number of hosts bitten by *Plasmodium*-infected mosquitoes (33). Likewise, trypanosome infection reduces salivary protein levels and increases probing frequency of infected tsetse flies (34), increasing the probability of effective parasite passage. Except in the case of *Leishmania* parasites, the mechanisms by which parasites alter probing frequency are not understood and nor is the process by which, for example, salivary protein production is reduced. Although fascinating, these are very difficult questions to answer experimentally.

The combination of challenges encountered by parasites within their vector causes bottlenecks that restrict the parasite genotypes in different tissues of the vector. Oberle *et al.* (35) recently quantified the frequency of marked lineages of African trypanosomes during tsetse transmission and discovered that few parasites successfully traverse from the midgut to the salivary gland, at least in laboratory infections. This bottleneck would be of little consequence in clonal populations; however, *T. brucei* can undergo sexual exchange in the salivary gland. Although sex apparently occurs infrequently in the field, its impact may be significant because different parasite lines show genetically determined virulence traits (36). Recombination in a mixed tsetse infection could generate new parasite lines with quite different disease progression in mammals.

When sex occurs in parasites within the arthropod vector, site and mechanisms can differ. For apicomplexan parasites such as *Plasmodium* and the cattle parasite *Theileria*, zygote formation occurs upon entry into the vector, whereas in African trypanosomes, sexual exchange precedes exit. Do these differences reflect where selection pressure for diversity is exerted, or are they simply an evolutionary relic? The mechanism can also vary. Hence, Mendelian inheritance has been established for *Plasmodium* and *T. brucei*, but for *T. cruzi* there is cell fusion of diploid parents and subsequent genetic loss (37), similar to the parasexual events of some fungi. It is not yet clear whether the recent discovery (38) of sexual exchange in *Leishmania* parasites in sandflies is conventionally Mendelian, but, like sex in the African trypanosomes, it is a rare event.

Outlook

The transmission cycles of vector-borne parasites are now among the most actively studied areas of parasite biology. This impetus has been enabled by developments in parasite culture, genetic analysis through genome sequencing, and through progress in the genetic manipulation of both the parasite and vector. Moreover, the increased sensitivity of detection for both transcripts and proteins at stages in the parasite life cycle where there are severe limitations in the biological material available have enabled previously cryptic events, such as sexual exchange, to be observed and studied. When combined with our increasing

understanding of the immune mechanisms operating in invertebrate vectors, analyses of the detailed interactions between parasites and their vectors are now tractable (Box 1).

Several themes are emerging. First, parasites do not passively transit through their life cycles in either the mammalian host or their vector. Instead, they are constantly surveying their environment, and it will be important to identify the signals and signaling pathways that they exploit and to understand the implications of these for their life history strategies.

Second, it is clear that parasites manipulate their vectors. Impaired feeding responses can improve the probability of parasite transmission and demand an understanding of how vector feeding behavior can be manipulated by the parasite. Here, the increasing sensitivity of mass spectrometry may allow parasite-released products to be identified within subcompartments of the vector and candidate molecular manipulators identified.

Third, parasites are likely to have fitness costs for their vectors. Filarial nematodes, for example, create considerable tissue damage as they migrate through their insect vector, with an impact on their longevity. Nonetheless, most parasites take time to mature within their vectors before becoming transmissible to a new host. Consequently, it is important that their vectors survive for long enough for parasites to mature and are not so disadvantaged that transmission is unlikely. Hence, parasites may minimize their impact sufficiently to maximize their probability of transmission or to generate compensatory fitness benefits, for example, by reducing the reproductive burden or increasing the defensive behavior of their vectors (33). These effects are difficult to study and hard to

quantitate, but nonetheless likely to have important consequences for transmission in the field.

Lastly, the co-evolution of parasites and their vectors has generated quite precise specificity in vectorial competence. This complicates genetic and functional studies of parasite-vector interactions, where the available laboratory strains of parasites and vectors may not be well matched. Nonetheless, understanding the restrictions of vectorial competence for a parasite has major implications for the transmission of parasites in different geospatial settings, directly affecting the likely success of particular intervention programs. Further, it is an essential component of predicting the probability of an expansion in a parasite's host range and the consequent emergence of new disease foci, as evidenced by the escape of *Trypanosoma evansi* from a dependence on tsetse flies, allowing it to spread through Asia and South America (39). For parasites with different ranges of host and vector specificity, the rapid developments in the tools for population genomic analyses of parasites and their vectors and hosts will prove highly informative for understanding parasite transmission potential in the field.

Although only a few details of the molecular events underlying parasite development within their vectors have been determined, transmission represents a vulnerable and attractive point of attack for preventing disease spread. Here, genetic determinants of parasite refractoriness, the impact of bacterial symbionts, and the mechanisms of parasite environmental sensing and developmental adaptation all offer important potential targets. Very often, the key molecules in the parasite will be highly specific and distinct from those in related parasites with different developmental cycles or vectors. For this reason, the most interesting discoveries may emerge from the analysis of the ~60% or more of parasite genes for which no function can yet be assigned. Hence, the development of effective forward genetic screens or high-throughput reverse genetic screens represents exciting progress.

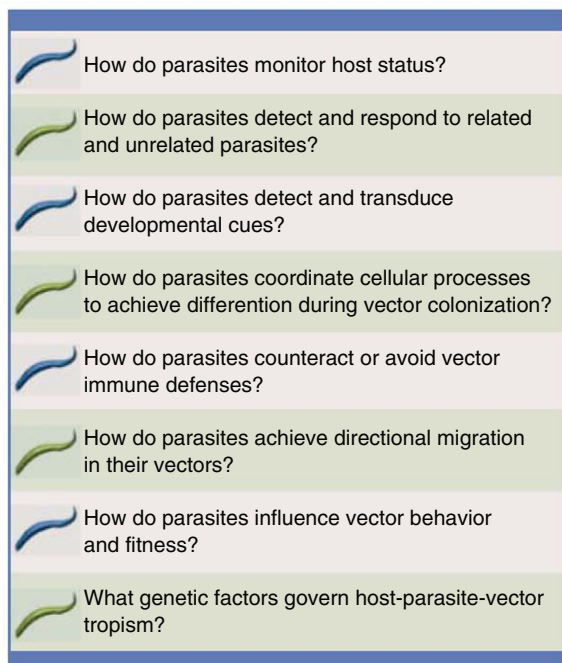
Nevertheless, strategies focused on manipulating the developmental cycles of parasites may have limited impact or even counterproductive effects. For example, the investment in transmission stages does not necessarily reflect the potential for transmission, particularly if low numbers of transmissible forms are sufficient for successful passage and establishment in the vector. Several studies have also revealed how parasites can modify their life histories in response to intervention, either to enhance transmission potential, virulence in the host, or pathology. Hence, mechanistic approaches alone may not be sufficient to predict the outcome of therapeutic strategies.

Instead, the integration of molecular study and evolutionary and mathematical analysis of an intervention's likely implication are necessary to devise effective and safe approaches to preventing disease transmission.

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Box 1. Key research questions relating to the transmission of vector-borne parasites.

Dynamics of Dpp Signaling and Proliferation Control

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Morphogens, such as Decapentaplegic (Dpp) in the fly imaginal discs, form graded concentration profiles that control patterning and growth of developing organs. In the imaginal discs, proliferative growth is homogeneous in space, posing the conundrum of how morphogen concentration gradients could control position-independent growth. To understand the mechanism of proliferation control by the Dpp gradient, we quantified Dpp concentration and signaling levels during wing disc growth. Both Dpp concentration and signaling gradients scale with tissue size during development. On average, cells divide when Dpp signaling levels have increased by 50%. Our observations are consistent with a growth control mechanism based on temporal changes of cellular morphogen signaling levels. For a scaling gradient, this mechanism generates position-independent growth rates.

Growth regulation of the *Drosophila* wing imaginal disc critically depends on the Dpp morphogen gradient (1–7). Dpp mutant imaginal discs fail to grow, and ectopic expression of Dpp in clones of wing cells organizes growth and elicits the formation of an ectopic winglet (7). Growth of imaginal discs is spatially homogeneous. How a graded Dpp signal can control homogeneous tissue growth is an open question for which a number of models have been proposed: For example, it has been suggested that the steepness of the gradient (5, 8) and/or mechanical feedback (9, 10) control proliferation. However, little quantitative data supports these models. To address this, we quantified spatial and temporal changes of Dpp concentration, signaling activity, and disc growth parameters during development.

The Dpp gradient scales with wing size. We used a functional green fluorescent protein–Dpp (GFP–Dpp) fusion (11, 12) expressed in the endogenous Dpp source to quantify GFP–Dpp profiles as a function of distance x from the source at different times t during larval development (Fig. 1, A to C), both with and without expression of the endogenous Dpp gene (13) (fig. S1). During the growth period, the Dpp gradient expands: Both the gradient amplitude C_0 (i.e., the concentration at the source boundary) and the decay length λ (the distance λ over which the gradient decays) increase significantly (Fig. 1, D and E). The decay length, λ , is proportional to the target tissue width L [the scaling ratio $\lambda/L = 0.112$ is

constant; Fig. 2, A and B; $n =$ two independent data sets with $\lambda/L = 0.107$ ($n_1 = 98$ discs) and $\lambda/L = 0.116$ ($n_2 = 60$ discs); table S3]. Further analysis of Dpp gradient profiles, $C(r,t)$, where $r = x/L$ is the relative distance to the source, revealed that the relative concentration gradient, $C(r,t)/C_0(t)$, is invariant during development (Fig. 2A); the gradient scales with the growing tissue. Gradient scaling behaviors have been reported in this and other systems (14–17), and possible mechanisms have been discussed (18, 19) [supporting online material (SOM) text S1.2]. Note that the gradient of another morphogen, Hedgehog (Hh), does not scale (fig. S2).

Decreasing degradation accounts for gradient expansion. Gradient expansion is not due to stretching of the gradient by wing growth, because the Dpp degradation rate is much larger than the disc growth rate; the gradient renews itself faster than the tissue grows (SOM text S1.1). Hence, gradient expansion is due to changes in Dpp production (v), diffusion (D), or degradation (k) (12, 20) (SOM text S1.1). Estimation of these parameters by fluorescence recovery after photobleaching (FRAP) (12) and a reporter assay [SOM quantitative procedures (QP) 3] showed that Dpp production and diffusion vary only slightly during the growth phase (Fig. 2, C and D), whereas the degradation rate decreases substantially as $k \sim 1/A$ with increasing posterior compartment area A (Fig. 2E). This decrease of the degradation rate could account for the constant scaling ratio λ/L , because $\lambda = \sqrt{D/k}$ and $A \sim L^2$ (12, 21) (SOM text S1.2). Furthermore, the gradient amplitude, C_0 , also increases, because of the decreasing degradation rate and the widening of the Dpp source (w) (Fig. 2F) (SOM text S1.1). Thus, changes of the Dpp source width and trafficking (degradation rate) result in Dpp gradient expansion during growth.

Cells experience an increase in Dpp concentration. Gradient expansion implies that cellular Dpp concentration changes over time. Two fac-

tors determine the cellular Dpp concentration: changes of the gradient profile (Fig. 1) and changes in cell position, $x_{\text{cell}}(t)$, in the growing tissue. Proliferation is approximately homogeneous in space (22, 23) (figs. S3A and S4A), so the relative position of a cell, $r_{\text{cell}} = x_{\text{cell}}(t)/L(t)$, remains constant as the tissue grows (fig. S3A; SOM QP5). Because r_{cell} is constant and the relative concentration gradient $C(r,t)/C_0(t)$ is invariant (Fig. 2A), the relative cellular concentration, $C(r_{\text{cell}},t)/C_0(t)$, is constant during development. Therefore, the average cellular Dpp concentration, $C_{\text{cell}}(t) = C(r_{\text{cell}},t)$, increases proportionally to the gradient amplitude, $C_0(t)$ (fig. S4C).

The Dpp concentration increases, on average, by 40% during each cell cycle. Does the increase in cellular Dpp concentration correlate with changes in the proliferation rate? We determined the proliferation rate (fig. S5; SOM QP4) from the area growth rate, $g = \dot{A}/A$, where $\dot{A}$ is the time derivative of the area A . This is a good approximation for the cellular proliferation rate because the cell density only shows a minor increase during wing growth (fig. S5, B and D). During the growth phase, the growth rate (g) decreases (fig. S5D), which reflects an increasing cell doubling time θ ($\theta \approx \ln 2/g$; SOM QP1), mostly because of a lengthening of the G_2 phase (24) (fig. S6).

We found that area growth correlates with the increase of the gradient amplitude by a power law (Fig. 2G)

$$C_0(t) \sim A(t)^\beta \quad (1)$$

where $\beta = 0.59$ ($n =$ two data sets; table S3). The average cellular Dpp concentration, C_{cell} , is proportional to the amplitude C_0 (see above) and therefore, $C_{\text{cell}}(t) \sim A(t)^\beta$. Derivation of this expression with respect to time reveals a correlation of the average growth rate ($g = \dot{A}/A$) with average temporal changes in the Dpp level ($\dot{C}_{\text{cell}}$) perceived by cells: $C_{\text{cell}}/C_{\text{cell}} = \dot{C}_{\text{cell}}/C_{\text{cell}} = \beta(\dot{A}/A) = \beta g$. Because the area growth rate and the cellular proliferation rate g_{cell} are approximately equal (see above), it follows that

$$g_{\text{cell}} \approx \frac{1}{\beta} \frac{\dot{C}_{\text{cell}}}{C_{\text{cell}}} \quad (2)$$

i.e., the proliferation rate is proportional to relative temporal changes of Dpp.

To estimate the relative increase of the cellular Dpp concentration $\alpha = \Delta C_{\text{cell}}/C_{\text{cell}}$ during the cell cycle time θ , we combine Eq. 2 with the approximations $\dot{C}_{\text{cell}}/C_{\text{cell}} \approx (\Delta C_{\text{cell}}/\theta)/C_{\text{cell}}$ and $\theta \approx \ln 2/g_{\text{cell}}$, and obtain the following:

$$\alpha = \frac{\Delta C_{\text{cell}}}{C_{\text{cell}}} \approx \beta \ln 2 \quad (3)$$

Thus, we find a constant $\alpha = 0.41$ ($n =$ two data sets; table S3); throughout development, cell division correlates with an increase of Dpp concentration by 40%.

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Dpp signaling activity parallels Dpp concentration. Proliferation depends on Dpp signaling activity, rather than ligand concentration (3, 25–27). We therefore measured Dpp pathway activity at different levels [reviewed in (28)]: phosphorylated Mad (P-Mad) (29), P-Mad/Medea complex formation, and *brk* and *dad* transcription [Fig. 3A and fig. S7; SOM experimental procedures (EP) 1] (30, 31). Of these, we systematically analyzed nuclear red fluorescent protein expressed under control of the *dad* enhancer (*dad*-nRFP) as a transcriptional readout reflecting cellular signaling activity, S_{cell} .

With time-lapse analysis, we confirmed that Dpp signaling increases in living wing discs (Fig. 3B and movie S1). Consistent with Eq. 2, relative changes in signaling, $\dot{S}/S$, are larger at early times of development, when growth is faster. Quantification of *dad*-nRFP profiles, $S(r,t)$ (Fig. 3C), in fixed discs showed that (i) the signaling gradient scales (Fig. 3D), i.e., the scaling ratio λ_s/L is constant (Fig. 3E); and (ii) the amplitude

S_0 increases with A as a power law (Eq. 1), with $\beta_s = 0.69 \pm 0.02$ (SEM) ($n = \text{four data sets}$; table S3), corresponding to $\alpha_s = 48\% \pm 2\%$ (Fig. 3F). Invariance (scaling) of the relative signaling profile, $S(r,t)/S_0(t)$ (Fig. 3D), implies that the cellular signaling level is proportional to the amplitude ($S_{\text{cell}} \sim S_0$). The power-law relation between amplitude S_0 and area A (Fig. 3F) indicates that the proliferation rate correlates with the average relative temporal increase of Dpp signal, $\dot{S}_{\text{cell}}/S_{\text{cell}} = \dot{S}_0/S_0$ (as in Eq. 2):

$$g_{\text{cell}} \approx \frac{\ln 2 \dot{S}_{\text{cell}}}{\alpha_s S_{\text{cell}}} \quad (4)$$

Here, $\alpha_s = 48\%$ implies that the cellular Dpp signaling level S_{cell} increases by about 50% during each cell cycle. On the basis of Eq. 4, we propose a model of growth control where the cell cycle length is determined by how fast an increase of cellular Dpp signal by 50% is achieved.

In different growth regimes, $\alpha_s \approx 50\%$. Dpp source and transport parameters contribute to the amplitude S_0 (SOM text S1.1) and therefore to cellular signaling levels S_{cell} . To test how the rate of increase of the gradient amplitude affects growth, we analyzed three conditions with changed Dpp source and/or transport parameters (SOM EP2): (i) haltere discs, where we found that Dpp production, diffusion, and degradation are smaller (32, 33) (Fig. 2, C to F; SOM QP3.2); (ii) wing discs with a Dpp source of haltere histotype (*dpp>Ubx*) (32, 33); and (iii) wing discs with a constant one-cell-wide source [limiting Hh signaling range to one cell with membrane-tethered Hh (Hh-CD2)] (34).

In these tissues, the decay time of the growth rate, the growth period, and final size differ from that of the wild-type wing disc (table S2 and fig. S8). However, growth and Dpp signaling still are related by the same features: (i) Gradients scale with tissue size. The scaling ratio λ/L is constant,

Fig. 1. Dpp gradient parameters. (A) *dpp-Gal4/UAS-GFP-Dpp* wing (Wi), leg (Le), and haltere (Ha) discs at different developmental times; w , source width, L , target width. (B) Images of GFP-Dpp gradients, corresponding to boxed areas in (A). (C) Quantification of GFP-Dpp concentration as a function of the distance to the source (x). (D and E) (D) Amplitude, C_0 and (E) decay length, λ , over time. At the end of the growth phase, in prepupal discs ($t > 140$ hours), C_0 again decreases. Error bars correspond to standard errors (SEM) of averages from binned data, and one data set per graph is shown. For fit functions, parameters, number of data sets, and number of discs per data set, see tables S1 to S3 and SOM QP1. For extended versions of figure legends, see SOM.

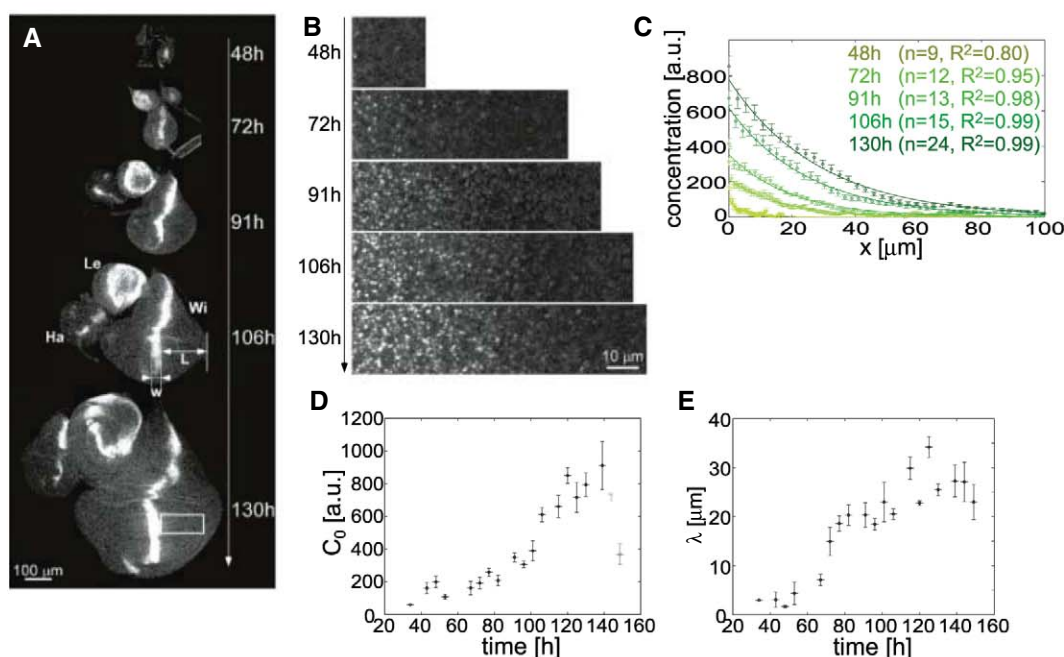


Fig. 2. The Dpp gradient and growth. (A) Relative Dpp concentration profiles $C(r, t)/C_0(t)$ from 48 hours to 130 hours (spanning the whole growth period) with density plot (below). Note the data collapse of gradient profiles onto a single curve. (B) Decay length, λ , versus compartment width, L . (C to F) (C) Dpp production rate, v ; (D) diffusion coefficient, D ; and (E) degradation rate, k , versus posterior compartment area, A ; and (F) Dpp source width, w , versus posterior compartment width, L , of wing (black) and haltere (blue) discs during growth, estimated by FRAP (red rectangles, wing) and a reporter assay (circles) (SOM QP3). (G) Gradient amplitude, C_0 , versus posterior compartment area, A .

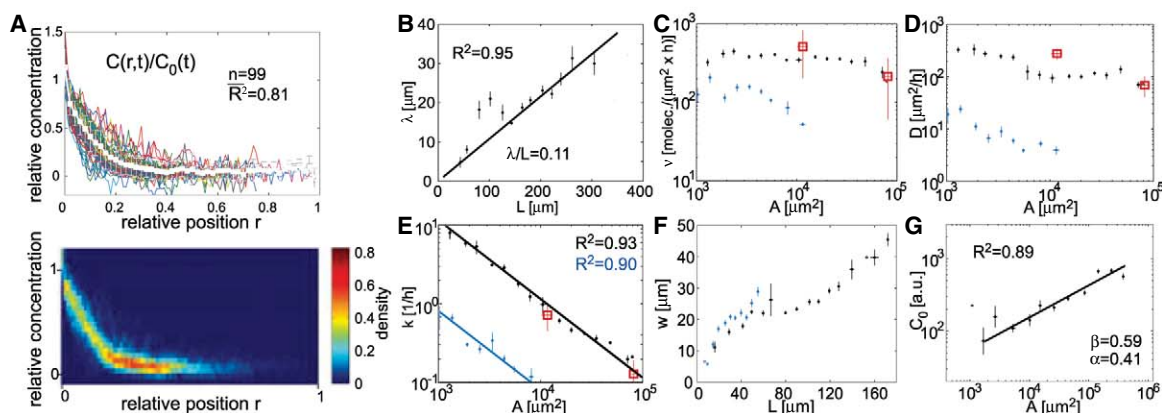
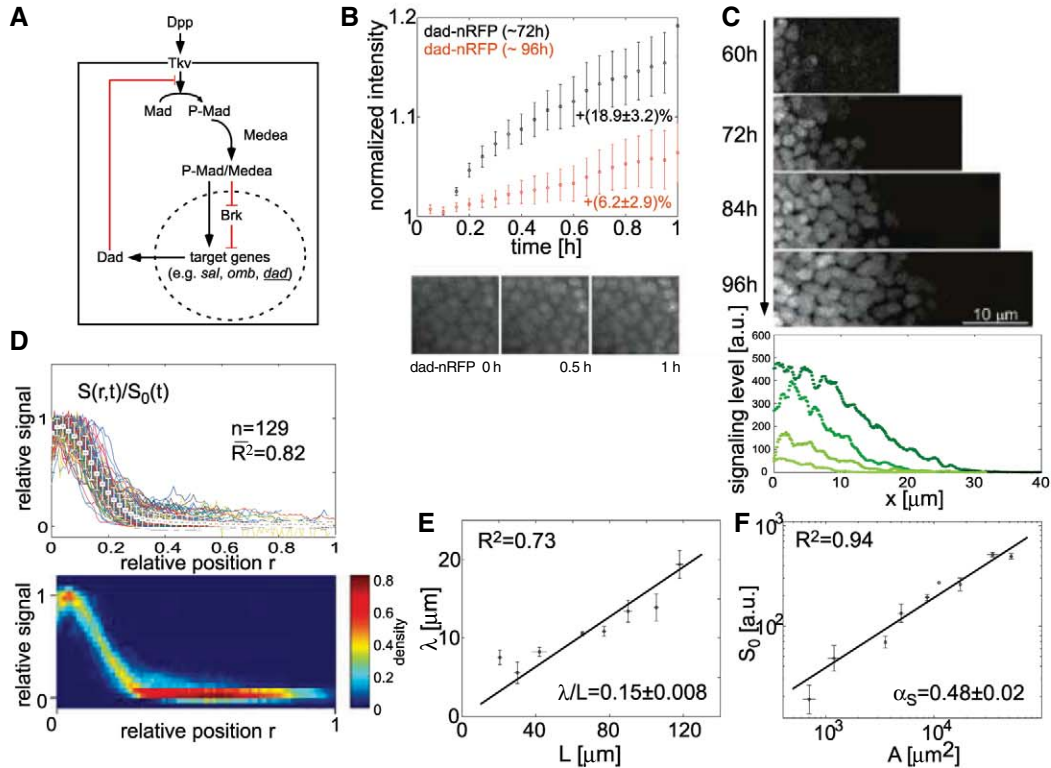


Fig. 3. Dpp signaling gradients. (A) Dpp pathway. **(B)** Average dad-nRFP intensity over time for time-lapse series at 72 and 96 hours of development (SOM QP6). Below, frames from a time-lapse series. **(C)** dad-nRFP images with quantification (60-hour and 72-hour images are contrasted). **(D)** Relative signaling profiles, $S(r, t)/S_0(t)$, spanning the whole growth period, with density plot. Profiles collapse onto a single curve. **(E and F)** (E) Decay length, λ , versus L and (F) amplitude, S_0 , versus A .



but different, in the different conditions (Fig. 4A and fig. S9), the relation between decay length and area is the same in all conditions except Hh-CD2 (Fig. 4C; SOM text S1.2). This points to a possible role of Hh for scaling. (ii) g and $\dot{S}_{\text{cell}}/S_{\text{cell}}$ are proportional during growth (Fig. 4B). And (iii), α_s is similar under all these conditions (Fig. 4D; mean of $\alpha_s = 49\% \pm 2\%$). Thus, cells divide when Dpp signaling levels have increased by about 50%, regardless of histotype (haltere versus wing), Dpp transport dynamics, or Hh signaling in the source.

A scaling mutant shows inhomogeneous growth while $\alpha_s \approx 50\%$. In all conditions discussed, the gradient scales, and proliferation is spatially uniform. In contrast, proliferation is not homogeneous when Dpp is ubiquitously expressed with the C765-Gal4 driver (C765>Dpp): Lateral positions have a larger growth rate (27). We quantified Dpp signaling and growth parameters in C765>Dpp to determine whether $\dot{S}_{\text{cell}}/S_{\text{cell}}$ is related to the local inhomogeneous growth rates in a manner consistent with Eq. 4.

In C765>Dpp discs, the spatial signaling profiles $S(r, t)$ increase over time, but do not scale (Fig. 5, A and B). We quantified the inhomogeneities of the growth rate, $g(r, t)$, using spatial profiles of phosphorylated histone H3 (PH3)-positive mitotic cells (Fig. 5C and fig. S3B; SOM QP4 and QP5). From $g(r, t)$ and $S(r, t)$, we then estimated g_{cell} , $\dot{S}_{\text{cell}}/S_{\text{cell}}$, and α_s for cells at different positions, $r_{\text{cell}}(t)$ (Fig. 5; SOM QP6). We noted that cells divide as they do in wild type when Dpp signaling levels have increased by about 50%, independent of time and cell position (Fig. 5D). Lack of scaling causes position

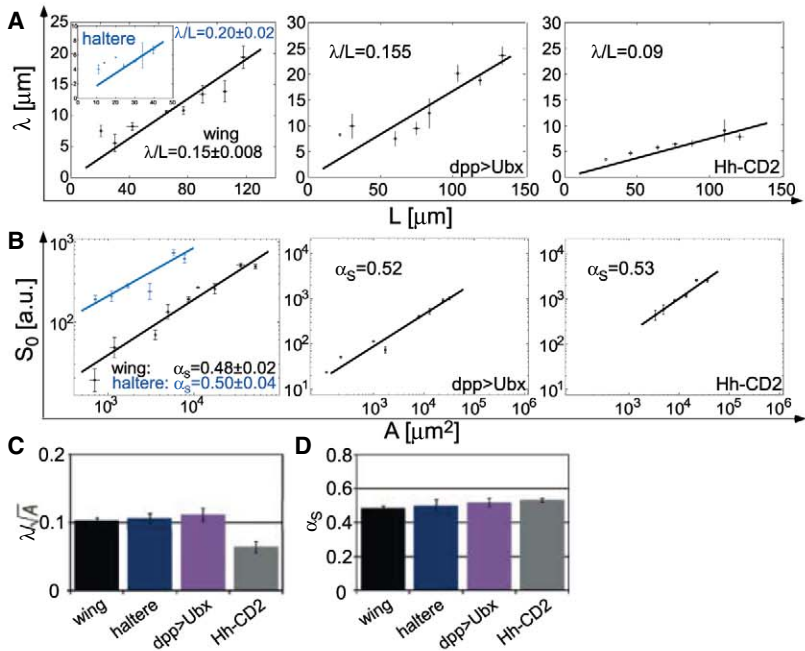


Fig. 4. Dpp signaling and growth in different growth conditions. (A and B) (A) dad-nRFP decay length, λ , versus L and (B) amplitude, S_0 , versus A for wing and haltere discs ($n = \text{four data sets}$; table S3); “haltere-wing chimera” (dpp>Ubx; $n = 2$); and the “one-cell-wide source” experiments (Hh-CD2; $n = 2$). **(C and D)** (C) Scaling ratio $\lambda/\sqrt{A}$ and (D) coefficient α_s .

dependence of $\dot{S}_{\text{cell}}/S_{\text{cell}}$ (SOM text S1.3), and thus, higher lateral growth rates are explained by the fact that the relative increase α_s is reached faster (i.e., $\dot{S}_{\text{cell}}/S_{\text{cell}}$ is larger) in lateral positions.

Exogenous manipulation of $\dot{S}_{\text{cell}}/S_{\text{cell}}$ results in predictable growth rates. To further test whether cells divide when Dpp signaling

levels increase by 50%, we used an established method to conditionally express the constitutively active Dpp receptor, Tkv^{QD}, in cell clones (5) (Fig. 6). Here, Tkv^{QD} transcription is induced exogenously, only after adding the progesterone-analog drug mifepristone (RU486) (5) (SOM EP2). In our model, exogenous manipulation of $\dot{S}_{\text{cell}}/S_{\text{cell}}$

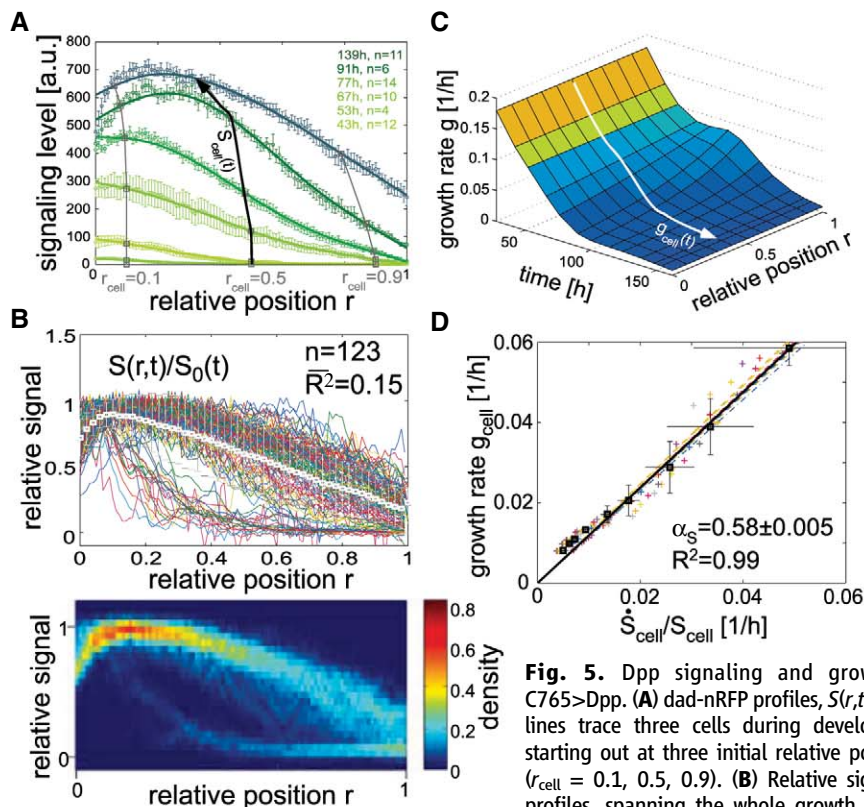
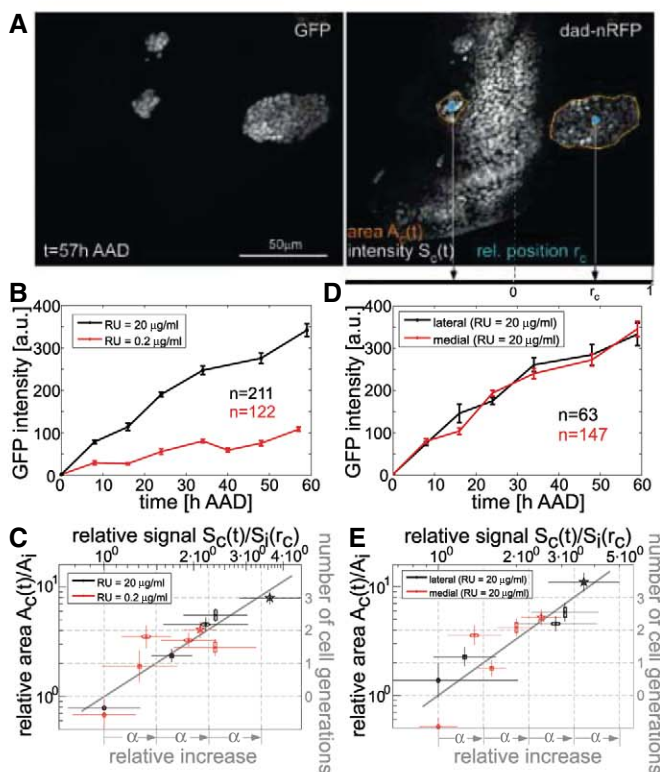


Fig. 5. Dpp signaling and growth in C765>Dpp. **(A)** dad-nRFP profiles, $S(r,t)$; black lines trace three cells during development starting out at three initial relative positions ($r_{\text{cell}} = 0.1, 0.5, 0.9$). **(B)** Relative signaling profiles, spanning the whole growth period, with density plot. Profiles do not collapse onto a single curve. **(C)** Spatiotemporal growth profile, $g(r,t)$; white arrow traces cell starting out at $r_{\text{cell}} = 0.5$. **(D)** Growth (g) versus $\dot{S}_{\text{cell}}/S_{\text{cell}}$ of cells starting out at different relative positions ($r_{\text{cell}} = 0.1, \dots, 0.9$; different colors).

Fig. 6. Tk v^{OD} clones (SOM EP2/QP7). **(A)** (Left) Tk v^{OD} clones marked by GFP; (right) dad-nRFP; relative position r_c , area A_c and average signaling level S_c of clones were measured in discs at different times after Tk v^{OD} induction [hours after addition of drug (h AAD)]. Genotype: $y w \text{hsFlp/UAS-p35}; \text{dad-nRFP/UAS-GFP}; \text{act}>y\text{Gal4}; \text{PR/UAS-Tkv}^{\text{OD}}$. **(B and D)** Average GFP intensity, reflecting Tk v^{OD} and p35 expression level for (B) different drug concentrations and (D) medial versus lateral clones. **(C and E)** Relative increase in clone area with respect to the initial area, A_c/A_i , versus relative increase in Dpp signaling levels, $S_c/S_i(r_c)$. For determination of A_i and S_i , see SOM QP7. Symbols in (C) and (E) indicate data points at different times AAD. Gray line (our model): For every signaling increase by $\alpha_S = 50\%$, clone area is expected to double, i.e., the cell generation number increases by 1.



in clones should result in clonal growth rates quantitatively predicted by Eq. 4.

For an RU486 concentration of 20 $\mu\text{g/ml}$, Gal4 activity (and, therefore, Tk v^{OD} expression) increases 3.6 times as fast as at 0.2 $\mu\text{g/ml}$; i.e., in these two experiments, $\dot{S}_{\text{cell}}$ is different (Fig. 6B). The relative increase in signaling levels in clones, $\dot{S}_{\text{cell}}/S_{\text{cell}}$, therefore, depends on RU486 concentration, as well as on the initial endogenous signaling level: Although exogenously imposed $\dot{S}_{\text{cell}}$ is the same in medial and lateral regions of the disc (Fig. 6D), clones in lateral positions experience a bigger relative increase in signaling upon Tk v^{OD} induction because their initial signaling level is lower, i.e., $S_{\text{cell}}/S_{\text{cell}}$ is dependent on clone position (fig. S10). If growth is causally controlled by $\dot{S}_{\text{cell}}/S_{\text{cell}}$, then RU486 concentration, clone position, and duration of drug exposure should determine the clone area, whereas α_S should be independent of these parameters.

The growth rate of clones indeed correlates with the exogenously imposed $\dot{S}_{\text{cell}}/S_{\text{cell}}$; i.e., the relative increases in clone area and in signaling level are correlated by a power law (Fig. 6, C and E; SOM QP7). The data are remarkably consistent with $\alpha_S \approx 50\%$, independently of RU486 concentration, duration of exposure (Fig. 6, B and C), and clone position (Fig. 6, D and E). Thus, local exogenous changes of $\dot{S}_{\text{cell}}/S_{\text{cell}}$ locally result in proliferation rates predicted by Eq. 4.

Simulation of growth control. To further test whether Eq. 4 describes a plausible growth mechanism, we developed a two-dimensional physical description of proliferation control [Fig. 7 and movies S2 to S5; SOM simulation (SI)]. We implemented Dpp and Hh production, diffusion, and degradation in a discrete vertex model that describes cells as polygons and accounts for the mechanical properties of the tissue (35). Dpp source cells are selected in response to Hh; Dpp production and diffusion are kept constant; and the Dpp degradation rate is dynamically altered in response to cell division events, such that the average degradation rate in the tissue becomes inversely proportional to the cell number (consistent with Fig. 2, C to F). A cell divides when the relative increase of the local Dpp level reaches a threshold α .

Simulations of this model using parameter values estimated experimentally for wing and haltere discs result in growth dynamics that quantitatively match the experimental observations for wing and haltere discs (Fig. 7, D to F, and movie S3), although only the initial cell number, minimal cell cycle length, and Dpp diffusion and degradation are different in the haltere. Furthermore, simulations of our model can account for the growth properties of Tk v^{OD} clones: Consistent with previous experimental results (3, 5), simulated lateral Tk v^{OD} clones have a bigger growth rate and grow to a larger size than medial Tk v^{OD} clones (about 4-fold as large as simulated wild-type clones, respectively) (Fig. 7, G to I), and cells surrounding Tk v^{OD} clones overproliferate (Fig. 7G; SOM text S2). These

results indicate that growth control by means of relative changes in Dpp levels could underlie growth control during imaginal disc development.

Conclusion. We have shown here that, in the wild type, Dpp concentration and signaling gradients scale and that, on average, cells divide when Dpp signaling levels have increased by $\alpha_s \approx 50\%$. A growth mechanism based on relative changes in signaling levels can quantitatively account for growth dynamics of wing and haltere discs, for inhomogeneous growth observed in scaling mutants, and for growth properties of TkV^{QD} clones. Other growth-control mechanisms we considered—for example, mech-

anisms based on spatial differences in Dpp concentration or signaling—are less consistent with our data (SOM text S1.3.2). However, the growth rule proposed here remains to be verified on the single-cell level.

Would the growth mechanism proposed here work in the entire wing? In wild-type discs, our Dpp concentration and signaling measurements were only significantly above background in medial regions. However, the $\text{C765} > \text{Dpp}$ and TkV^{QD} clone experiments show that the $\bar{S}_{\text{cell}}/\bar{S}_{\text{cell}}$ mechanism also works laterally. Furthermore, in our simulations of wild-type disc growth, Dpp molecule numbers in lateral regions are low but

sufficient to provide enough precision to control proliferation by a temporal growth rule (SOM SI8). The simulations indeed capture the main growth properties of the complete wing and haltere, which indicates that the growth mechanism could in principle operate globally.

How could cells determine relative increases in Dpp signaling? Sensitivity of signaling systems to relative changes of input is typical for adaptive sensory systems and plays a role in bacterial and sperm chemotaxis and in olfactory and visual transduction, where determination of relative temporal changes is achieved by combining adaptation with a dynamic response (36–40). The

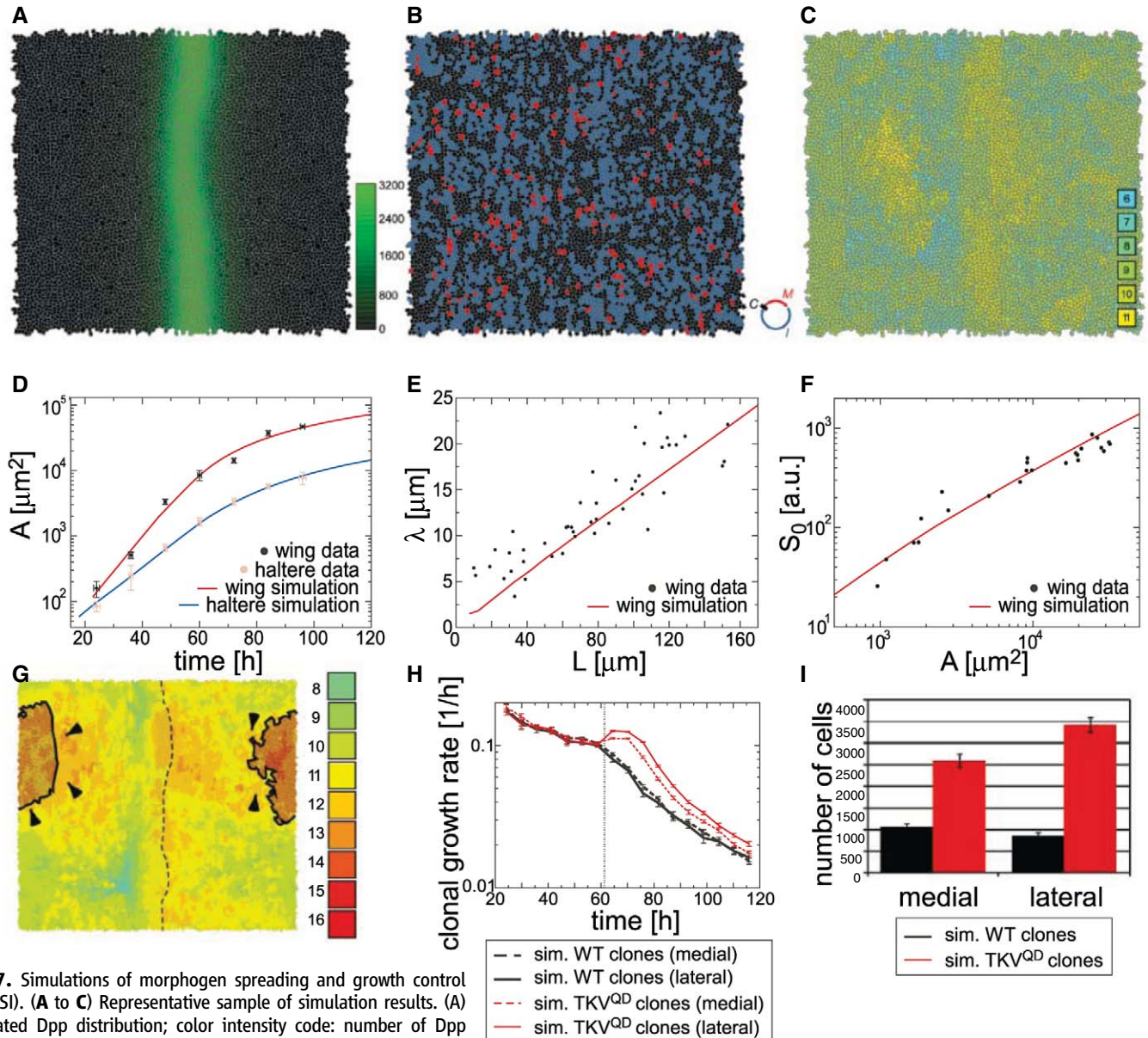


Fig. 7. Simulations of morphogen spreading and growth control (SOM SI). (A to C) Representative sample of simulation results. (A) Simulated Dpp distribution; color intensity code: number of Dpp molecules. (B) Cell cycle state [red, M (M phase); blue, I (constant phase in interphase); black, C (checkpoint phase)]. (C) Cell generation number (color-coded). (D to F) Averaged simulation results (red and blue, $n = 25$) with experimental data (black and gray). (D) Posterior compartment area over time. (E) Decay length versus posterior compartment width. (F) Gradient amplitude versus posterior compartment area. (G to I) Numerical simulations of wild-type and TkV^{QD} clones located close to and far from the source

(“medial” and “lateral,” respectively). (G) Lateral clone expressing TkV^{QD} (black outline; dashed line, AP boundary) with cell generation number (color-coded); note nonautonomous effects on proliferation around the clone (arrowheads). (H) Average growth rates of simulated medial and lateral TkV^{QD} (red) and control (black) clones ($n=25$); vertical dashed line: induction of TkV^{QD} production; I, average simulated clone size at $t = 120$ hours.

Dpp pathway is a dynamic network and may generate adaptive responses by combining feed-forward and feedback elements in its structure. Consistent with this idea, in *brk^{X4}* mutants, where the Dpp network is perturbed, α_s has a different value (fig. S11) [This mutant is discussed in detail in SOM text S2.4].

Our observations raise the question of what molecular mechanisms underlie (i) cell cycle control via relative temporal changes of Dpp signaling, (ii) gradient scaling via decrease of Dpp degradation, and (iii) final size determination. Although pupariation time and final size were different in the different conditions studied, the average cell cycle length at pupariation was about 30 hours for all (table S2; SOM text S1.3.4), which suggested that cells could stop dividing when their cell cycle is prolonged beyond a threshold. Response to this threshold could be deregulated in some tumor mutants.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/331/6021/1154/DC1

Materials and Methods

SOM Text

Figs. S1 to S53

Tables S1 to S5

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Movies S1 to S5

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Crystal Structure of the Dynein Motor Domain

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Dyneins are microtubule-based motor proteins that power ciliary beating, transport intracellular cargos, and help to construct the mitotic spindle. Evolved from ring-shaped hexameric AAA-family adenosine triphosphatases (ATPases), dynein's large size and complexity have posed challenges for understanding its structure and mechanism. Here, we present a 6 angstrom crystal structure of a functional dimer of two ~300-kilodalton motor domains of yeast cytoplasmic dynein. The structure reveals an unusual asymmetric arrangement of ATPase domains in the ring-shaped motor domain, the manner in which the mechanical element interacts with the ATPase ring, and an unexpected interaction between two coiled coils that create a base for the microtubule binding domain. The arrangement of these elements provides clues as to how adenosine triphosphate-driven conformational changes might be transmitted across the motor domain.

The cytoskeletal motor proteins consist of the myosin family, which moves along actin filaments, and the kinesin and dynein families, which move along microtubules. These motors use a common principle to generate movement in which they bind to their track, undergo a force-producing conformational change, release from the track, and then return to their original conformation. These structural changes are

coupled to chemical transitions in the motor's adenosine triphosphatase (ATPase) cycle [adenosine triphosphate (ATP) binding, hydrolysis, and product release].

The force-generating cycles of kinesins and myosins are understood in considerable mechanistic detail. Even though they interact with different cytoskeletal polymers, kinesins and myosins share a protein fold, reflecting their common evo-

lutionary origin (1). Dyneins, by contrast, are unrelated to kinesins/myosins and instead have evolved from the AAA family of ATPases (2). The AAA ATPases (ATPases associated with diverse cellular activities), which are present in both prokaryotes and eukaryotes, participate in diverse functions, including protein unfolding for proteolysis, disassembly of stable protein complexes, and helicase activities (3). The majority of AAA ATPases self-assemble into hexameric rings that carry out the functional activities of these enzymes (4, 5). Dynein is one of two AAA ATPases that has six distinct AAA domains concatenated within a single polypeptide chain [the other being Rea1, an ATPase involved in ribosome biogenesis (6)]. Electron microscopy (EM) studies of dynein have shown that these AAA domains fold into a ring-shaped structure similar to other AAA ATPases (7–9).

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Dyneins can be divided into three major families: axonemal dyneins, which power the beating of cilia and flagella; intraflagellar transport (IFT) dyneins, which transport proteins in the axoneme; and cytoplasmic dyneins, which perform most of the minus-end-directed transport of cargos (e.g., membranes, mRNAs, nuclei, and viruses) along microtubules in eukaryotic cells (10, 11). The N-terminal third of the >500 kD dynein heavy chain (Fig. 1A), which differs considerably between each dynein subfamily, contains elements that confer oligomerization (IFT and cytoplasmic dyneins are dimers, and axonemal dyneins can be monomers, dimers, or trimers) and binding sites for accessory chains and cargos. The remainder of the heavy chain contains the motor domain, which has a conserved sequence across the dynein family. The N-terminal region of the motor domain is termed the “linker” and has been proposed to serve as a mechanical element because its position shifts in different nucleotide states (7, 8, 12). The linker is followed by four AAA domains (AAA1 to 4) that contain functional nucleotide binding sites. ATP hydrolysis at AAA1 is essential for motility, whereas the role of nucleotide binding/hydrolysis at AAA2 to 4 is less clear but appears to influence motility (13–15). AAA5 and AAA6 have lost their conserved nucleotide binding sites and may serve a structural role (2). Unlike kinesin and myosin, where the polymer and nucleotide binding sites are in close proximity (<4 nm), dynein binds to microtubules through a small globular domain at the end of a 15-nm-long antiparallel coiled-coil stalk that emerges between AAA4 and AAA5 (16–18) (Fig. 1A). Because microtubule binding stimulates ATP turnover at AAA1 and the nucleotide state of AAA1 affects microtubule binding affinity, allosteric changes must be communicated over long distances and across multiple domains within the motor.

In comparison with kinesin and myosin, dynein’s mechanism of motility is poorly understood, in part because of the lack of high-resolution structural information. Here, we present the crystal structure of the dynein motor domain in the context of a 610-kD dimer of two motor domains.

Structure determination. We expressed recombinant *Saccharomyces cerevisiae* cytoplasmic dynein in yeast (19) using fed-batch fermentation (20). To identify a compact and monodisperse protein that might crystallize, we varied the N-terminal truncation of the linker and removed different lengths of the coiled-coil stalk. A truncated motor domain fused to glutathione S-transferase (GST) (Fig. 1A) produced small crystals in a screen, which were subsequently optimized (20). With the exception of the removal of the microtubule binding domain (MTBD), this GST fusion corresponds to a previously characterized dimeric motor (GST-Dyn1_{314kD}) that moves processively and at similar velocities to the wild-type yeast dynein holoenzyme (19). The unit cell (174 by 119 by 203 Å) of the diffracting crystals contained one dynein dimer and had a

P2₁ space group (see table S1 for crystallographic information and statistics). To obtain phases, polytungsten clusters (W12) were incorporated into the crystals, and phases were determined to 8 Å by two-wavelength anomalous diffraction and extended to 6 Å by solvent flattening (20). The experimental phase information produced an interpretable electron density map with clear densities for helices and β sheets (fig. S1); the electron densities of the two dynein motor domains are nearly identical to one another, with minor differences in the clarity of a subset of loops connecting secondary structural elements. At this resolution, side chains cannot be resolved; hence, a polyalanine polypeptide chain was built into the electron density map. Due to the conserved structure of AAA domains, the majority of secondary structural elements predicted by sequence could be assigned in the model (uncertainties in assignment and connectivity exist for the linker and C-terminal domains, which do not have good homology models) [see supporting online material (20) for details of model building and caveats]. Because secondary structure predictions and our electron density map are both imprecise, registry errors of several amino acids will be common throughout our model. However, the primary focus of this study is to characterize the domain organization of the dynein motor.

Structural overview of a dynein dimer. The two dynein motor domains, which are dimerized by GST, are related by a pseudo-two-fold symmetry, such that the linker-face of the AAA rings appose each other and the stalk domains

point in opposite directions (Fig. 1B). The presence of the linker domain makes it unlikely that the two AAA rings can directly stack on each other, as has been observed in other AAA proteins (21). The motor domain is a ring-shaped structure with a central hole; viewed from the top, the ring is somewhat asymmetric, with one side curved and the other more flattened (Fig. 1, B and C). The overall appearance is similar to negatively stained EM images of an inner arm axonemal dynein from *Chlamydomonas* (7) and a cytoplasmic dynein motor domain from *Dictyostelium* (8) (fig. S2). The linker arches over the center of the AAA ring (we refer to this side of the ring as the “linker face”) (Fig. 1, B and C). In previous EM images, it was thought that the linker might wrap around the top of the ring (7), but the crystal structure shows that the two ends of the linker contact the ring and the central portion bows well above the central hole like the handle of a basket (Fig. 1B and fig. S1B). The two contact points of the linker, adjacent to the base of the stalk and across the ring by AAA1, are consistent with the EM images of dynein in the nucleotide-free (apo) state (8) (fig. S2). Two coiled coils emerge from the ring, one of which corresponds to the “stalk” that extends to the MTBD. The second coiled coil (which we term the “buttress”) is predicted in the sequence (22). This uncharacterized coiled coil might correspond to the side of a triangular structure that has been visualized in a subset of the EM images (fig. S2B) (7). The C-terminal helices lie on the side of the ring opposite from the linker

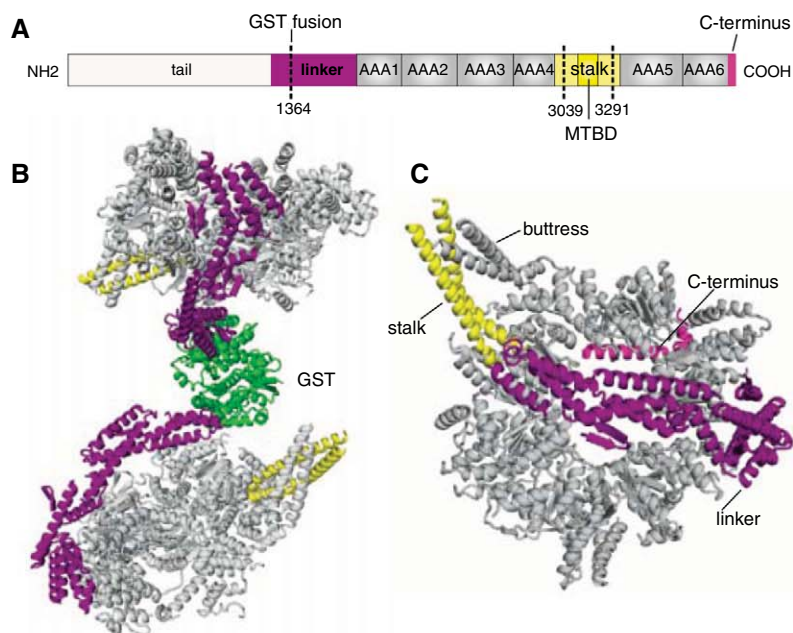


Fig. 1. The cytoplasmic dynein motor domain crystal structure. (A) Schematic illustrating the domains of the dimeric yeast cytoplasmic dynein heavy chain. For crystallization, the linker was fused to GST, and the MTBD and part of the stalk (amino acids 3039 to 3291) were removed and replaced with a short peptide (20). (B) The complete GST-cytoplasmic dynein dimer; GST is in green. (C) View of the motor domain from the linker face. The linker, stalk, and C terminus are color-coded as in (A).

(Fig. 1C) in agreement with EM studies (fig. S2); we refer to this face of the ring as the “C-terminal face.”

Structure and assignment of the AAA domains.

The six AAA domains can be easily identified in the electron density map (overview in Fig. 2A). AAA proteins possess an α/β large domain, which contains the nucleotide binding Walker A (P-loop) and B motifs, and a helical bundle small domain that extends from the C terminus of the large domain (Fig. 2B). The large domain (AAAL) is characterized by a parallel five-strand β sheet surrounded by two α helices (H0 and H1) on one face and three helices (H2, H3, and H4) on the opposite face, although different subclasses of AAA proteins contain unique insertions to this core structure (4). In our map of both motor domains, the AAA domains show a nearly identical pattern of electron density (fig. S3A), with five helices (H0 to H4) in similar positions to other AAA proteins (Fig. 2C and fig. S3, B and

C). H0 and H1 in the six AAA domains are on the exterior of the ring, as in other hexameric AAA proteins. Although individual β strands are difficult to identify, a diffuse electron density in the position and orientation expected for the AAA β sheet is observed (Fig. 2C and fig. S1). When viewed from the side, the large domains form the top layer of the linker face of the ring.

The six dynein AAA small domains (AAAs), which form the C-terminal face of the ring, all show a similar pattern of five helices (H5 to H9) (Fig. 2E and fig. S4A), whose connectivity can be established by well-ordered loops. The general placement of the small domains in the ring and connectivity of H5 to H8 are similar to NSF (N-ethylmaleimide-sensitive fusion protein) (fig. S4C) and several other AAA proteins. Dynein has evolved a fifth helix (H9) at the bottom of the small domain, which helps to connect the small domain of one AAA domain to the large domain of the neighboring AAA in the poly-

peptide chain. In contrast to an earlier suggestion (4), the extra helix in dynein is at the C terminus rather than the N terminus of the small domain; thus, the small-domain architecture is not similar to the BclI subclass of AAA proteins. AAAs 5 and 6 contain additional helices beyond H9, although their connectivity is not well established in our model. The stalk and buttress coiled coils are extensions of small-domain helices (fig. S4A).

Several pieces of information enabled us to assign AAA1 to 6 within the electron density map. First, we could use information from previous EM studies. The apo form of dynein seen by EM is strikingly similar to our crystal structure (fig. S2, A and B); the N terminus of the linker and the stalk base create fiducial markers that allow the x-ray and EM structures to be aligned with one another. The order of the AAA domains around the ring has been determined by EM after tagging particular domains with engineered fluorescent protein markers (8). When thus aligned, the order of the AAA domains is clockwise around the ring when viewed from the linker face, with AAA1 positioned close to the linker C terminus based on sequence (Fig. 2A). In the model presented in this EM study, however, the linker was assigned to the opposite face of the AAA ring, possibly because of insufficient three-dimensional discrimination.

Further evidence for assigning the individual AAA domains came from the inspection of the electron density maps for characteristic insertions to the canonical large domain AAA core structure. AAA1, AAA2, AAA3, and AAA5 have β -hairpin insertions between H3 and β 4, a feature that is found in a subset of other AAA proteins (4) (Fig. 2, B and D, and fig. S5, A and B). AAA2 has an additional loop insertion within H2 (fig. S5A); a similar combination of H2 and H3 inserts is found in NtrC (23) (fig. S3B) and BclI (24) but few other AAA proteins. AAA4, 4, and 5 have an extra N-terminal helix that packs against H0 and H1, as observed in certain other AAA proteins such as ClpX (fig. S3C). All of these features in the dynein sequence (fig. S5B) could be observed in the electron density maps (fig. S3) and helped to make our domain assignments.

The AAA small domains also have unique characteristics that could be matched to the electron density maps. Our AAA domain assignment places the stalk and buttress coiled coils within the small domains of AAA4 and AAA5, respectively, in agreement with their position in the sequence (fig. S5B). In addition, AAAs H5 to H8 are predicted to be longer than in other AAA domains, which is seen in the electron density map (fig. S4A) and agrees with an alignment of our crystal structure to the EM of *Chlamydomonas* axonemal dynein (7) (fig. S2B). When viewed from the linker face, the small domains are located clockwise with respect to each large domain, as is true of many other AAA ATPases such

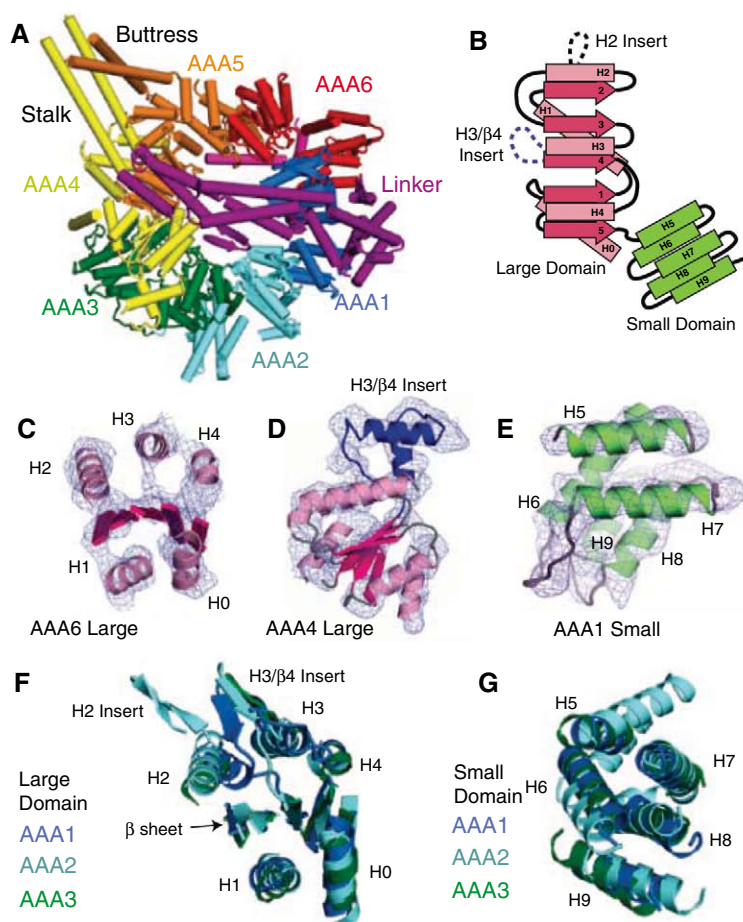


Fig. 2. The dynein AAA domains. (A) The six individual AAA domains are highlighted in colors. The linker spanning over the center of the ring is magenta. (B) Topology of the dynein large and small domains. (C) The electron density map [experimental (F_o) map contoured at 1 σ] and model build of the secondary structure elements (helices and central β sheet) of a dynein AAA large domain (AAA6L). (D) Experimental electron density map and model showing a unique helical insert (blue) in AAA4L. Insert sequences are shown in fig. S5. (E) Experimental electron density map and model of a dynein small domain (AAA1s). Omit maps of AAA1 and AAA2 small domains are shown in fig. S1. (F and G) Comparison of the large and small domains of AAA1 to 3. Galleries for all large and small domains can be found in figs. S3 and S4, respectively.

as ClpX (25) and HslU (26). Of the six AAA domains, AAA1 and 3 small and large domains are the most similar to one another in structure, as is true of their sequences as well.

To further validate our assignment, we obtained information on methionine positions using selenomethionine (SeMet). SeMet was incorporated into our yeast-expressed dynein to 64% occupancy, using a modification of previously described methods (20, 27). Although our 7.5 Å SeMet data was not sufficient to calculate phases, we were able to combine it with our experimentally determined phases to generate an anomalous difference Fourier density map in which we could locate approximate positions of 64 of the 126 methionines (peaks at $>3\sigma$) in the GST-dynein dimer (table S1 and fig. S6). Although only four sites (out of 18 total methionines) were observed in the poorly ordered GST molecule, their locations corresponded to known methionine positions (fig. S6). Within the dynein motor domain, 42 sites were located in similar positions in each motor domain (21 in each monomer), whereas the remaining 18 sites were only observed in one of the two motor domains. Some of these peaks corresponded to predicted Met locations based on our model (fig. S6B). However, due to the low-resolution SeMet map, it was not possible to use this information to establish a registry of the polypeptide chain. Thus, although the secondary structure assignments in our model (fig. S5B) are likely to be mostly correct, the precise registry of the residues in our model will not be accurate (20). However, by counting the number of SeMet peaks in each of the large or small AAA domains, we could determine whether these numbers were consistent with our assignment of the six AAA domains. In the assignment shown in Fig. 2A, the number of SeMet peaks was equal to or less than that expected from the dynein sequence (fig. S6C). On the other hand, if the AAA assignment was shifted by one in either the clockwise or counterclockwise direction, then mismatches occurred, in which more SeMet peaks occurred in several domains than would be predicted from the sequence (fig. S6C). Thus, the SeMet data, the positions of large and small domain insertions, and agreement with previous EM mapping all support the AAA assignment shown in Fig. 2A.

Distinct conformations of the AAA domains produce an asymmetry of ring. The arrangement of AAA domains around the motor domain ring is more asymmetric than any AAA hexamer structure solved to date. Viewed from the side, the AAA large domains occupy different planes within the ring (Fig. 3A). Viewed from the linker face, larger gaps are evident between the large domains of AAA1 and AAA2 and between AAA5 and AAA6 (Fig. 3B). Two major factors determine the positions of the AAA domains. First, as noted for other AAA proteins (25, 26), a flexible linker between $\beta 5$ of the large domain and H5 of the small domain allows for rigid body rota-

tions between these domains (Fig. 3C and fig. S7). Second, the small domains all pack rigidly and in a similar orientation against the large domains of the neighboring AAA (e.g., AAA1s against AAA2L) (Fig. 3D and fig. S7), as was noted for ClpX (25). Thus, AAA(*n*)small-AAA(*n*+1)large may behave as rigid units that can move relative to one another by rotations about the peptide linker that connects the large and small subdomains within a AAA domain.

Hexameric AAA enzymes bind ATP between adjacent AAA subunits, with the clockwise neigh-

bor [viewed from the linker face (Fig. 2A)] contributing residues that promote nucleotide hydrolysis (4, 5). In other AAA proteins, the close apposition of large domains enables nucleotide binding and hydrolysis (28, 29), whereas a greater separation is associated with a nucleotide-free or apo state (25, 30). In dynein, mutagenesis studies suggest that AAA3 can bind and hydrolyze ATP (13). Although we cannot ascertain whether nucleotide is bound to the AAA domains at our current resolution, AAA3 and AAA4 are positioned in a similar closed conformation to the interface

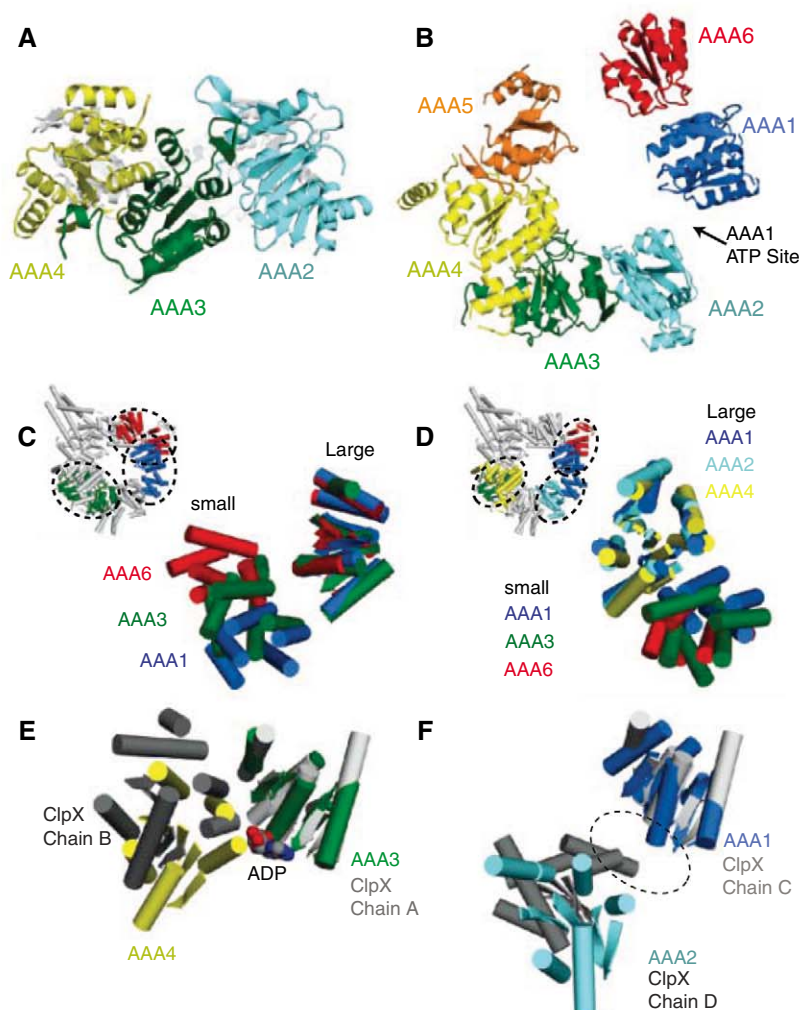


Fig. 3. Asymmetry of the dynein ring. (A) Side view of AAA ring showing the different planes occupied by the AAA1 to 3 large domains. (B) View of the linker face of the AAA ring (as in Fig. 2A), showing just the large domains. Note the large gaps between AAA1 and 2 and between AAA5 and 6. ATP is expected to bind between AAA1 and 2. (C) Comparison of the positions of the small domains relative to the large domain for AAA1, 3, and 6 (large domains aligned to AAA1); small domains can adopt a variety of different orientations due to a flexible linker joining $\beta 5$ (large) to H5 (small). (D) Packing of the small domains against the neighboring AAA large domain. (Inset) AAA1s against AAA2L, AAA3s against AAA4L, and AAA6s against AAA1L. AAA large domains were aligned to AAA1. The orientations of the small domains to the neighboring large domain are similar to one another. (E) Comparison of the positions of adjacent large domains in dynein and ClpX (PDB code 3HWS). In the nucleotide-bound ClpX monomers in the hexamer (monomer chain A shown here) and AAA3 of dynein (aligned with ClpX), the adjacent large domain is closely apposed in a closed conformation. (F) In the nucleotide-free monomers of the same ClpX hexamer (monomer chain C) and AAA1, the adjacent large domain is more widely separated in an open conformation, due to the rotation of the intervening small domain. Comparison of the positions of adjacent large domains around the dynein AAA ring is found in fig. S7.

of the nucleotide-bound subunits in the ClpX hexameric ring (Fig. 3E) (25). On the other hand, AAA1, the main hydrolytic site of dynein, is

separated by a large distance from AAA2, which is more similar to the interface of the nucleotide-free subunits in ClpX (Fig. 3F) (25). The con-

formation of AAA1 and AAA2, combined with our crystallization in a nucleotide-free buffer and the observed position of the linker domain, makes it likely that AAA1 is in an apo state. It seems highly probable that ATP binding will close the gap between AAA1 and AAA2 and help to trigger other conformational changes in the dynein ring.

The linker domain. The arching linker domain is composed of four predominantly helical subdomains (Fig. 4). The C terminus subdomain 4 is composed predominantly of a four-helix bundle that interacts with the large and small domains of AAA1, as well as part of the small domain of AAA6. This extensive interface is suggestive of a stable interaction. The N-terminal subdomain 1 has a pattern of α helices that is reminiscent of the repeating unit in spectrin (31). It contacts H2 and the H3- β 4 hairpin insert of the AAA5 large domain, which differs from previous suggestions that the linker N terminus interacts with either AAA2 (12) or AAA4 (8). The limited subdomain 1–ring contact raises the possibility that the N terminus of the linker might dissociate from AAA5 during the ATPase cycle. Subdomains 2 and 3 meet through long helices, which have a small region of overlap at their ends. The lack of extensive interactions between subdomains 2 and 3 and between subdomains 3 and 4 raises the possibility that one of these regions could act as a hinge to allow rigid body motions of subdomains 1 and 2 in other nucleotide states.

The stalk and buttress. In addition to the stalk coiled coil, our crystal structure revealed a second antiparallel coiled coil that emerges from the AAA5 small domain (Fig. 5, A and B) and corresponds to a region of strong coiled-coil prediction (22). It likely corresponds to a bridging structure noted by EM (fig. S2B) that was then assumed to be part of the stalk rejoining the AAA ring (8). Strikingly, the AAA5 coiled coil extends toward and makes contact with the stalk (Fig. 5B). Based on the position of overlap and length of the helices in the stalk, we postulate that a highly conserved tryptophan in CC2 and a glycine in CC1 of the stalk (18) are positioned in the region of overlap with the AAA5 coiled coil. Based on its potential role in supporting the stalk, we have named the AAA5 coiled coil the “buttress.” This term need not imply a completely static support, and indeed the buttress may influence the conformation of the stalk during dynein’s ATPase cycle.

The stalk and buttress coiled coils are extensions of helices in the small domains of AAA4 and 5, respectively, somewhat analogous to the insertion of an antiparallel coiled coil in the small domains of ClpB and Hsp104 (32). The two helices (CC1 and CC2) of the stalk coiled coil are extensions of the AAA4 helices H7 and H8, respectively, whereas the buttress coiled coil is an extension of AAA5 helices H5 and H6 (Fig. 5C). The stalk and the buttress both possess a kink close to their junctions with

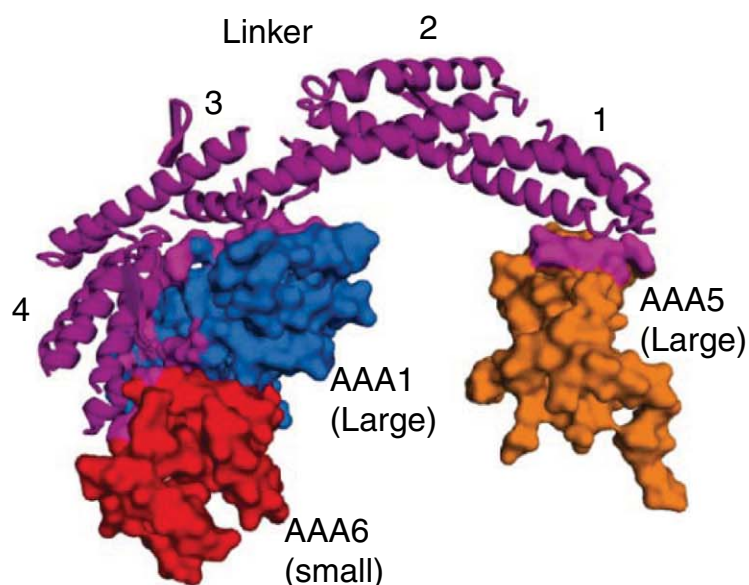


Fig. 4. The linker domain and its interaction with the AAA ring. The linker and its four helical subdomains (subdomains 1 and 4 are N- and C-terminal, respectively) are indicated, and the contact sites (<8 Å) with the AAA domains are shown in color on the space-filling model.

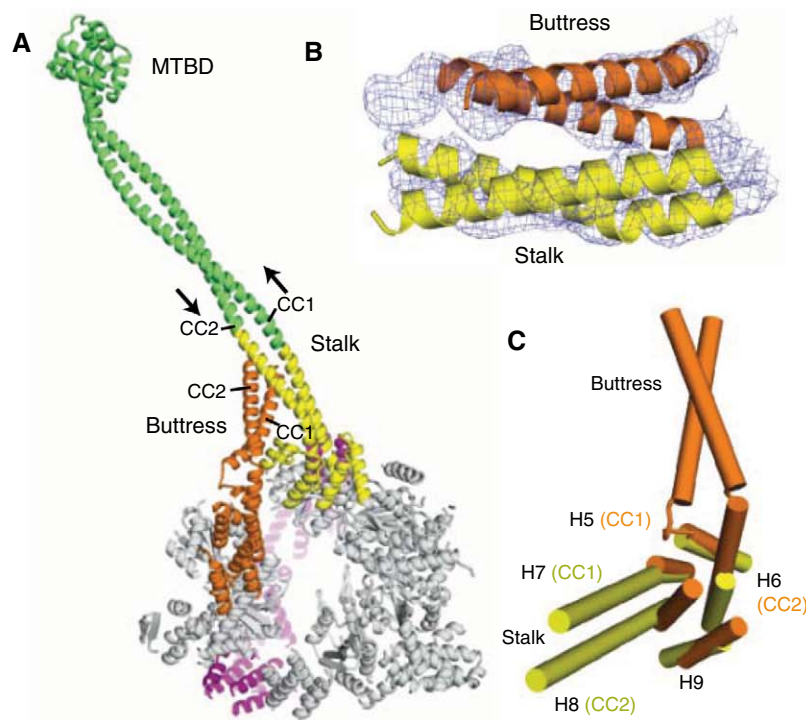


Fig. 5. The stalk and buttress coiled coils. **(A)** The motor domain, highlighting the stalk and buttress, viewed from the C-terminal face. The stalk from this crystal structure is highlighted in yellow, and the green extension is a continuation of the stalk modeled with an antiparallel coiled coil of the proper length. The MTBD and distal coiled coil is from a previously solved crystal structure (PDB code 3ERR). **(B)** The experimental electron density map (1 σ contour) and model showing the likely interaction of the distal part of the buttress with the stalk. **(C)** The small domains of AAA4 and AAA5 show that H7 and H8 extend into the stalk coiled coil, and H5 and H6 extend into the buttress coiled coil.

the core small domain (Fig. 5C), which causes their direction to deviate from that of the small-domain helices and allows the two coiled coils to interact.

The interaction between the stalk and the buttress provide new ideas for how the MTBD at the end of the stalk (Fig. 5A) might be regulated by the AAA ring. Previous work has suggested that the two helices (CC1 and CC2) in the stalk coiled coil might slide relative to one another, undergoing a half-heptad shift in different nucleotide states; this sliding would propagate ~10 nm to the MTBD and influence its polymer binding affinity (18, 33, 34). Our previous model suggested that CC1 might be pulled from its base, relative to a stationary CC2 (18). However, because CC1 and CC2 merge into the well-packed helices of the AAA4 small domain, it is unlikely that either helix can move at its base. The presence of the buttress, however, suggests another possibility. Through its contact with the stalk, the buttress might relay rigid body motions between AAA domains (e.g., between AAA4 and 5) into shear motions between the helices of the stalk coiled coil.

Allosteric communication. For dynein to function as a motor protein, nucleotide transitions at AAA1 (the main ATP hydrolytic site) must be conveyed to the linker and distant MTBD. The unexpected large gap between AAA1 and AAA2 provides a clue as to how such long-range allosteric communication might take place. In AAA proteins, ATP binds at the interface between adjacent AAA subunits, with each subunit contributing to nucleotide binding and hydrolysis (3–5). For nucleotide to bind and be hydrolyzed at AAA1, the AAA1 and AAA2 large domains must move toward one another (Fig. 6A), producing a closed conformation similar to that of AAA3–AAA4 and active nucleotide hydrolyzing sites of other AAA proteins (Fig. 3, E and F). Nucleotide binding would provide the energy that drives the closure of AAA1–AAA2, similar to the closing of the pocket that has been observed between the apo and nucleotide-bound states in the F1-ATPase (35, 36).

Closure of the AAA1–AAA2 gap by nucleotide binding could trigger a conformational change that propagates around the ring to reach the AAA4s/AAA5L unit. We speculate that this could be achieved by a domino effect, in which the closure of the AAA1–AAA2 gap induces the motions of adjacent rigid units (AAA2s/AAA3L, AAA3s/AAA4L, and AAA4s/AAA5L). Any movement in this last unit (AAA4s/AAA5L) will have the combined effect of applying tension to the binding interface between the stalk and the buttress, as well as between the linker and the AAA5 large domain. The nucleotide state of AAA2, 3, and 4 (not known in our structure) might subtly change the positions of these domains and thus regulate the transmission of the conformational change between AAA1 and AAA4s/AAA5L.

Important consequences of ATP-induced rigid body movements of AAA domains are a likely

conformational change in the stalk and the detachment of the linker from its contact with AAA5 large, which has been observed by EM (8). The fate of the linker after its detachment remains unknown. The H2 and H3/β4 inserts that lie on top of the large domains in AAA2, AAA3, and AAA4 (Fig. 3F) might provide secondary docking sites for the linker. Indeed, comparable inserts provide binding sites for the AAA helicase RuvB with RuvA (37) and the AAA transcriptional activator PspF with the σ^{54} subunit of RNA polymerase (23). Alternatively, once detached from AAA5, the linker might be mobile, as has been suggested by its positional variation seen by EM (8). Similar to the conformational change in the neck linker of kinesin

(1), a transition of the dynein linker from a detached or weakly bound “pre-powerstroke” state to a strongly, AAA5-docked “post-powerstroke” state (8) may constitute part of the mechanism for producing unidirectional motion.

Interaction of the dynein dimer with microtubules. Our structure of a GST-dimerized dynein provides insights and raises new questions concerning how cytoplasmic dynein binds to and moves along a microtubule. To move processively, myosin V, myosin VI, and kinesin bind with both motor domains (heads) to their tracks, at least for some portion of their motility cycles (38, 39). To enable simultaneous microtubule binding of both MTBDs, the two dynein motor domains must rearrange substantially from the

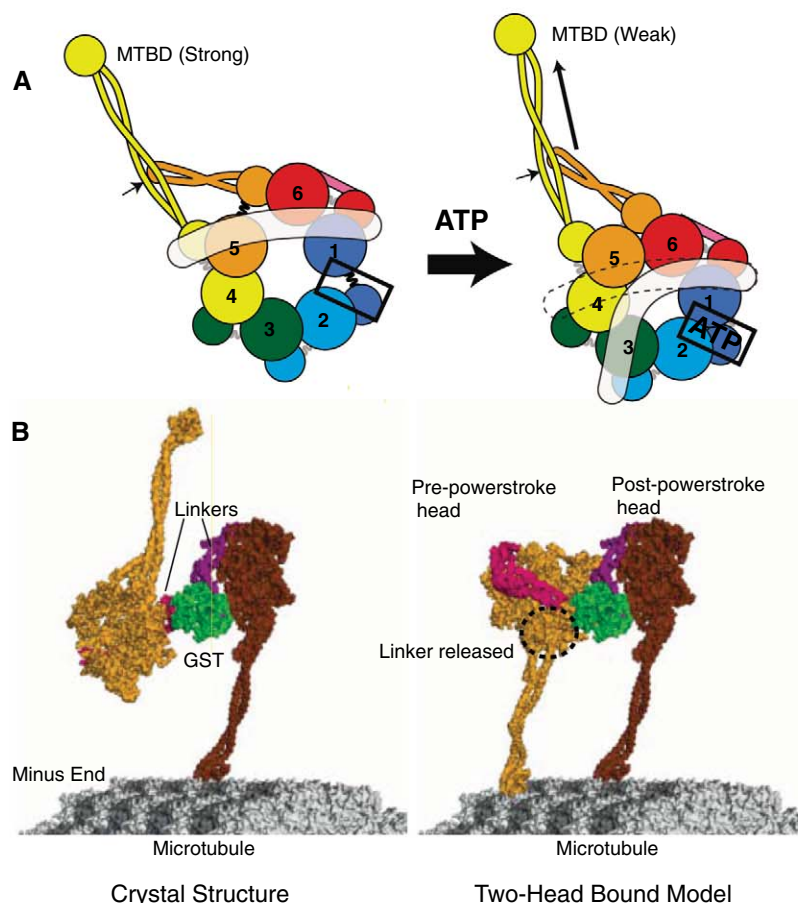


Fig. 6. Models for dynein conformational changes. **(A)** A schematic model showing how ATP binding to AAA1 might propagate a conformational change to the MTBD and linker (in white). (Left) The apo state with gaps between AAA1 and 2 and between AAA5 and 6, as based on our crystal structure (Fig. 3B). (Right) The proposed consequence of ATP binding to AAA1: closure of the AAA1–2 gap, which in turn pulls upon and moves AAA2, 3, and 4. The relative motions that might ensue between AAA4 and 5 cause the detachment of the linker from AAA5. The indicated position of the linker in the ATP state is based on EM studies of Roberts *et al.* (8), although it may be mobile and not have a defined docked state on the ring. AAA4 and 5 movement may create a shear between the stalk (yellow coiled coil) and buttress (orange coiled coil); if the stalk preferentially interacts with the buttress through one of its helices, this could shift the registry of the stalk helices (arrow) that propagates to the MTBD to change its affinity (18, 34). **(B)** (Left) The dynein crystal structure with the distal stalk and MTBD, modeled as in Fig. 5A. One of the MTBDs is docked onto the microtubule as determined previously (18). (Right) One possible model in which the second head (light orange) has its MTBD docked onto the microtubule. To allow this head and its MTBD to bind to the microtubule, its linker was undocked from AAA5 and rotated about subdomain 3, and a slight bend was applied to the stalk of the front (left) head.

conformation found in our crystal structure (Fig. 6B). Generating a two-head-bound microtubule intermediate cannot be achieved simply by a rotation around the GST-linker boundary. Moreover, the possible ways to position the two heads on the microtubule are constrained by a “short leash” between the GST and the motor domains (Fig. 1B). However, we could dock the second head to the microtubule and create a two-head-bound intermediate by rotating the motor ring, detaching the linker, and altering the stalk (Fig. 6B, right). In this tentative model, the MTBDs are bound to neighboring protofilaments of the microtubule, with one MTBD positioned 8 nm in front of the other (Fig. 6B). The front head with the detached linker is in a pre-powerstroke state, and the rear head with the docked linker is in a post-powerstroke state (7, 8), which is similar to the conformation of the mechanical elements of kinesin and myosin in their two-head-bound intermediate state (38, 39).

The two-head-bound model shown in Fig. 6B raises questions of how the rear MTBD advances past the front MTBD as dynein steps along the microtubule (19). Redocking of the linker in the front head would pull on the rear head, causing it to move forward after it dissociates from the microtubule. It is possible that the MTBD of the rear head also swings forward, through a rotation of the ring or a change in the stalk-ring angle, so that it can more easily rebind to a new tubulin binding site toward the microtubule minus end. Information on the relative positions of the ring, stalk/MTBD, and linker

in different nucleotide states will help to resolve the sequence of conformational changes that occurs as cytoplasmic dynein steps along the microtubule.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1202393/DC1
Materials and Methods
Figs. S1 to S7
Table S1
References

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REPORTS

Synthesis and Measurement of Ultrafast Waveforms from Five Discrete Optical Harmonics

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Achieving the control of light fields in a manner similar in sophistication to the control of electromagnetic fields in the microwave and radiofrequency regimes has been a major challenge in optical physics research. We manipulated the phase and amplitude of five discrete harmonics spanning the blue to mid-infrared frequencies to produce instantaneous optical fields in the shape of square, sawtooth, and subcycle sine and cosine pulses at a repetition rate of 125 terahertz. Furthermore, we developed an all-optical shaper-assisted linear cross-correlation technique to retrieve these fields and thereby verified their shapes and confirmed the critical role of carrier-envelope phase in Fourier synthesis of optical waveforms.

Function generators routinely produce electric fields in the form of sine, sawtooth, square, and triangular waves and even sophisticated arbitrary waveforms in the radio-frequency (RF) regime. These function generators use electronic oscillators whose speed is limited to ≤ 100 GHz to obtain the waveforms.

Extending the production of similar waveforms to the optical region (10^{15} cycles/s) is of great interest but presents a major challenge (1). By manipulating the phases of high-order harmonics generated in an intense laser field, researchers have obtained single-cycle attosecond sine and cosine pulses in the extreme ultraviolet to soft

x-ray region (2). Alternatively, by spectral (line-by-line) control of individual components of a comb of carrier frequencies, they can produce shaped envelopes that engulf many cycles of a rapidly oscillating optical field and have a high level of complexity and good fidelity (3). The challenge that we address here is the realization of instantaneous electric field optical waveforms similar to those obtained with function generators in the RF region and the measurement of these fields.

It is known from Fourier transform theory that periodic waveforms can be synthesized with a series of sine or cosine waves at frequencies starting with the fundamental frequency. When these frequencies span much more than an octave

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in the frequency spectrum, they form a train of subcycle pulses (4). For controllable manipulation of these waves, their initial phases must be stable, preferably aligned with each other; the phase slip between the peak of the synthesized field and the peak of the envelope that shrouds the field (the carrier-envelope phase or CEP) should remain constant.

An approach that could satisfy these conditions is to expand the spectrum of a mode-locked Ti:sapphire laser (5). Another is to link and phase-lock the spectra of two mode-locked lasers (6). However, pulse shapers with a resolution that is sufficiently high to provide octave-spanning line-by-line phase and amplitude control are not available. Another approach is to generate a wide comb of precisely equidistant optical frequencies by nonlinear mixing of phase-coherent laser oscillators, but the complexity of the locking scheme has thus far inhibited realization of this scenario (4).

Yet another approach that has demonstrated success in frequency comb generation is molecular modulation (7). In this method, two lasers with a frequency difference nearly equal to a molecular vibrational or rotational transition are used to adiabatically drive the molecular ensemble. With H_2 as the molecular medium, an equidistant comb of frequencies has been generated with an overall conversion efficiency of $>10\%$. The spectrum of the generated comb easily covers several octaves (8–11). Furthermore, by beginning with a driving laser frequency that is nearly equal to the molecular Raman resonance, the frequencies of the comb are commensurate ($\omega_n = n\omega_m$, where ω_m is the modulation frequency). The CEP is then stable and can be controlled precisely at all times (12, 13). With a comb thus generated, the aforementioned conditions for optical waveform synthesis are satisfied, permitting the synthesis of repetitive femtosecond and subfemtosecond optical waveforms of any desired shape.

Here we describe the synthesis of electromagnetic waves whose instantaneous electric fields are shaped to form a train of periodic sawtooth, square, or subcycle cosine and sine pulses. The fields we synthesized are enclosed under a Gaussian envelope of 3.5-ns full-width half-maximum (FWHM), by virtue of the pulsed laser we used to generate the frequencies used in the synthesis. The repetition rate of the pulses in the train is 125 THz, determined by the fundamental frequency, and is equivalent to a period duration of 8.02 fs. The shortest pulse synthesized within each period is a subcycle cosine pulse that has a field FWHM of 834 attoseconds and an intensity envelope FWHM of 1.45 fs. These results manifest a major step toward achieving the optical analog of a RF function generator.

The optical waveforms we synthesized have a multi-octave-spanning spectral width, and as a result are difficult to characterize with often-used ultrafast pulse characterization techniques (14). We show here that when the frequencies are commensurate, shaper-assisted cross-correlation can be effectively used to retrieve these fields and

thereby verify their successful synthesis. We used the technique to confirm the critical role of CEP in waveform synthesis.

To prepare the harmonic frequency comb, we directed a monochromatic beam of transform-limited linearly polarized pulses 3.5 ns in duration, with its frequency centered at 4155.235 cm^{-1} ($\sim 20\text{ mJ/pulse}$, 15 pulses/s), into a LiNbO_3 crystal to generate its second harmonic. Both colors were then used to drive the Q(1) vibrational Raman coherence of room-temperature H_2 at a pressure of 1 bar to produce several higher-order harmonics to form a frequency comb (13, 15). The power of the fundamental and the second harmonic exiting the cell were attenuated by a factor of 3 to avoid optical damage to the spatial light modulators and other optics downstream. The net pulse energies of the first five harmonics generated were 320, 270, 150, 33, and $6.7\text{ }\mu\text{J}$ ($\pm 20\%$ each, $\sim 780\text{ }\mu\text{J}$ total), respectively, measured just in front of a 200- μm -thick β -barium borate (BBO) crystal that mixes the harmonics to produce heterodyne signals used to adjust the phases and to set the CEP (Fig. 1A). The front face of this β -BBO crystal was where the shaped optical fields were realized and characterized.

When independently controlling the amplitude and the phase of each harmonic, the maximum number of distinguishable wave shapes that can be synthesized with N harmonics is 2^N . Fine variation of these 2^N waveforms is possible subject to the resolution, overall stability, and signal-to-noise ratio. To achieve the highest degree of complexity and fidelity, it is optimal to begin with N as large as possible. Yet simulation shows that commonly used periodic waveforms such as the square, sawtooth, subcycle sine and cosine, and triangle can be synthesized to a good degree of resemblance to the actual waveform with just the fundamental plus the next few harmonics (16). Hence, for demonstration purposes, we used the first five harmonics (first to fifth components of the frequency comb) to synthesize optical fields

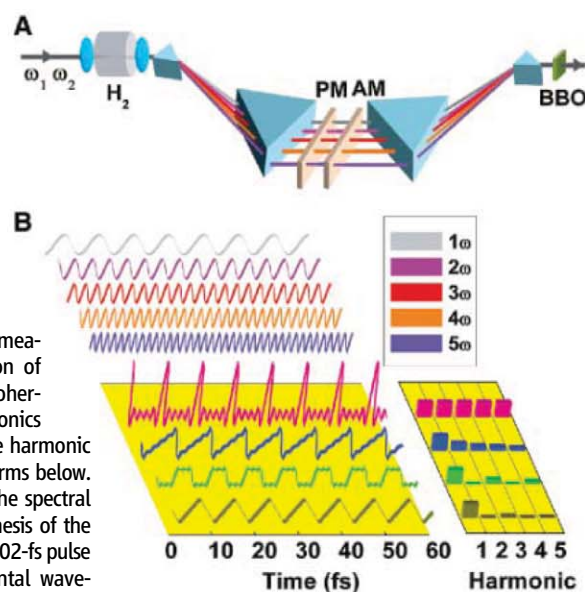
and verify their shapes in this work. The corresponding wavelengths used were nominally 2406, 1203, 802, 602, and 481 nm. With these five harmonics, the frequency comb spectrum extends over two octaves. The shortest pulses they can collectively synthesize are transform-limited subcycle cosine pulses, each spanning 0.75 optical cycle with a temporal field FWHM of 834 attoseconds. A few of the optical field waveforms that could be synthesized with these harmonics are depicted in Fig. 1B. Parameters relevant to their synthesis are tabulated in table S1.

After generating these five harmonics, we set their amplitudes to the desired values. The amplitude of each harmonic was attenuated by a home-built liquid crystal spatial light modulator (LCSLM) that was fabricated as an amplitude modulator. The next major step was to align their phases. Because the comb frequencies are multiples of the fundamental frequency, every harmonic will heterodyne with a signal at the same frequency derived by optically summing two lower harmonics. The resulting interference signal can then be used to align the phases of the comb frequencies and for phase compensation and optimization (4, 13). We followed the alignment procedure described in (13). The signal obtained from heterodyning the second harmonic generated from the β -BBO crystal with that from the Raman process was used to establish the CEP. To adjust the phases, we used a LCSLM configured for the task (15).

Once the phases are aligned and the amplitudes are set to values required for the desired waveform, the synthesis is complete. A train of pulses of a desired shape is created from the superposition of these harmonics. The instantaneous field of the pulses can be reconstructed from the known amplitudes, the CEP, and the alignment of the relative phases.

We next show that the fields can be measured to verify the waveforms. It has long been

Fig. 1. (A) Schematic of the experimental setup to synthesize waveforms using harmonics generated by coherent modulation of the H_2 molecule. ω_1 and ω_2 designate the frequencies of the inputs to the H_2 cell. AM and PM are liquid crystal spatial light modulators (LCSLMs) that, respectively, attenuate the powers (and thus amplitudes) of the harmonics and adjust and compensate their phases. These LCSLMs are also used as pulse shapers for the cross-correlation measurements. **(B)** Pictorial demonstration of ultrafast waveforms obtained by the coherent superposition of the first five harmonics of a fundamental wavelength. The five harmonic waves are depicted above, the waveforms below. The panel on the lower right depicts the spectral field amplitudes required for the synthesis of the respective waveforms on the left. The 8.02-fs pulse spacing originates from a fundamental wavelength of 2406 nm.



established that field retrieval is possible by linear cross-correlation (17). The method is seldom used because implementing it requires a reference pulse with an exceptionally broad bandwidth. In the present experiment, the bandwidth of more than two octaves is sufficient for linear cross-correlation to be employed. We used the electronic pulse shaping technique (18) to split the synthesized waveform $E(t)$ into a reference pulse waveform $E_1(t)$ to retrieve the field of a target waveform $E_b(t)$, where $E_b(t) = E(t) - E_1(t)$ (19). Because of this splitting, 100% retrieval of $E(t)$ is not possible. However, by choosing $E_1(t)$ to be small compared to $E(t)$, one can achieve nearly complete retrieval by this technique. Theoretical justification of this approach and a detailed description of its implementation are given in Materials and Methods of the supporting online material. The value of the relative phases among the harmonics is required in programming the pulse shaper, so these values

must be known when implementing electronic pulse splitting for this correlation measurement.

We begin by retrieving the field of a sawtooth waveform. The CEP of a sawtooth waveform is $\pi/2$, which we set at the time of the synthesis. Thus, $E(t) = \{i, i/2, i/3, i/4, i/5\}$. The energies of the first five harmonics were attenuated from their initial values by the amplitude-adjusting LCSLM to 167, 41.7, 18.5, 10.5, and 6.7 μJ per ns pulse, respectively. The total pulse energy of $\sim 244 \mu\text{J}$ thus comprises normalized amplitudes (which scale as the square root of energy) of $\{1, 1/2, 1/3, 1/4, 1/5\}$ for the harmonics, as required by a sawtooth waveform. To perform the cross-correlation, we created a transform-limited subcycle cosine pulse train $E_1(t) = \{1/10, 1/10, 1/10, 1/10, 1/10\}$ and $\phi_{n1} = 0$, $\phi_{\text{cel}} = 0$. The remaining field of the sawtooth then constitutes a second pulse train $E_b(t)$. $E_b(t) = E(t) - E_1(t) = \{i - 1/10, i/2 - 1/10, i/3 - 1/10, i/4 - 1/10, i/5 - 1/10\}$ (in the complex plane). These

phasor quantities of $E_1(t)$ and $E_b(t)$ were used to program a pulse shaper to split $E(t)$ into $E_1(t)$ and $E_b(t)$ and set a time delay τ between the two fields. The pulse shaper comprises the same phase and amplitude LCSLMs that were used in the first part of the experiment. Varying τ then produced the cross-correlation of $E_1(t)$ and $E_b(t)$. The cross-correlation signal $I(\tau)$ is the sum of the average power of the harmonics at each delay τ and was measured with a broadband thermopile without dispersing the harmonics, because the waveform materializes only when the harmonics are superimposed in time and space. $I(\tau)$ was then recorded and processed with a PC. Meanwhile, because the $I(\tau)$ measurement is an average power measurement that is insensitive to phase fluctuations, we verified that the CEP and the relative phases of the harmonics remained stable throughout the correlation measurement by measuring the heterodyne signal of the incident harmonics at random time delays during each correlation scan; we thereby ensured that the phases had not drifted and pulse splitting was properly executed so that the observed results could be interpreted correctly.

Histograms of the cross correlation signal taken at each time delay are shown in Fig. 2A. The associated relative-phase information of the incident field extracted from heterodyne measurements is shown in Fig. 2B. The observed changes to the relative phases at various time delays have a cumulative standard deviation of $\pm 0.05\pi$. This phase fluctuation only has a minor effect in distorting the shape of the waveform (fig. S3).

We simulated the cross correlation result using Eq. 1:

$$I(\tau) \propto \sum_n (a_0^2 + b_n^2) + 2a_0 \sum_n b_n \cos(n\omega\tau + n\phi_{bn} + \phi_{\text{cel}}) \quad (1)$$

where a_0 is the amplitude of $E_1(t)$, and b_n , ϕ_{bn} , and ϕ_{cel} are the amplitudes and the relative phases of the n th component and the CEP of $E_b(t)$. These amplitudes and phases are the normalized phasor quantities of $E_1(t)$ and $E_b(t)$, and their values are based on those of normalized $E(t)$ shown above. The simulation and the measured data are connected by a linear scaling factor and a DC offset, which we obtained by least-squares fitting of the calculated result to the measured data at each delay τ . The fitted factor and offset in turn are

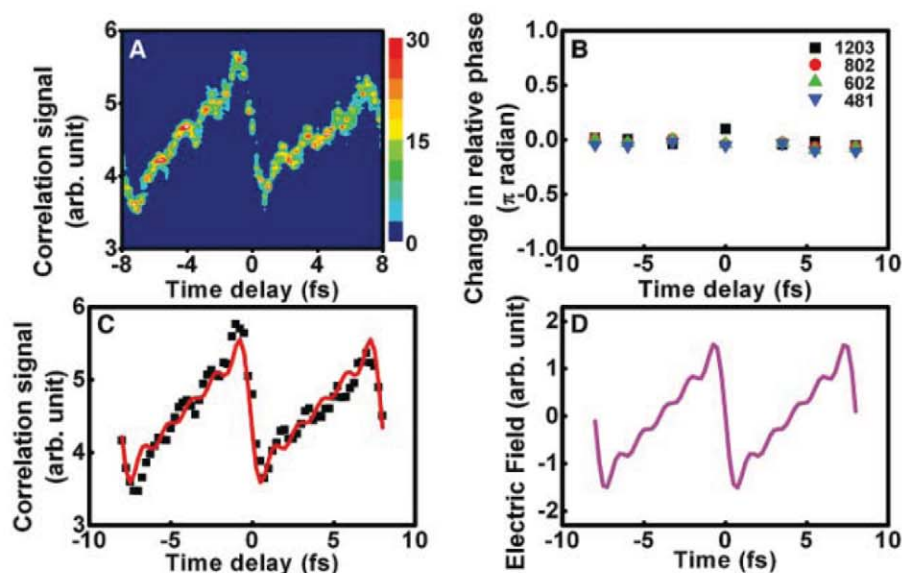


Fig. 2. (A) Color-coded histograms of the measured linear cross-correlation signal. A third axis (color scale) represents the frequency of occurrence of the measured signal strength at each time delay. (B) Time-averaged fluctuation of the relative phase of the incident harmonics derived from heterodyne signals at each of the harmonics measured at randomly selected time delays and plotted as a change in the relative phase. Each data point is the average of 30 laser shots. (C) Numerical simulation (solid red curve) scaled to match the measured correlation data (points). The method of scaling is explained in the text. Each data point is the average of 120 readings of the thermopile meter recording. At zero time delay, the waveform in front of the β -BBO crystal is a sawtooth. This waveform is Fourier reconstructed with the measured CEP and the amplitudes of the five harmonics and is shown in (D) for comparison with the signal in (C).

Fig. 3. (A to C) Evolution of the cross-correlation signal when only the CEP (not harmonic field amplitude) is varied in synthesizing the waveform. The normalized amplitudes are the same as in Fig. 2. The CEP values are shown in the respective panels. These waveforms all have the same envelope, but their instantaneous electric fields as represented by the cross-correlation signal are substantially different, in complete agreement with Fourier Transform theory. The corresponding reconstructed fields are shown in the insets as a reference.

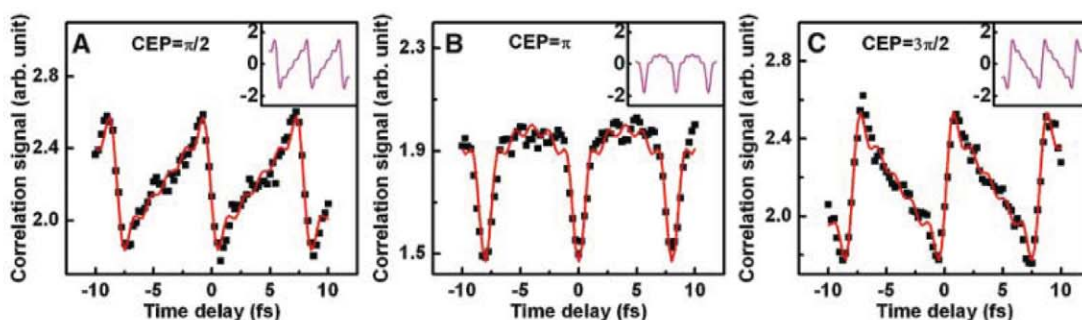
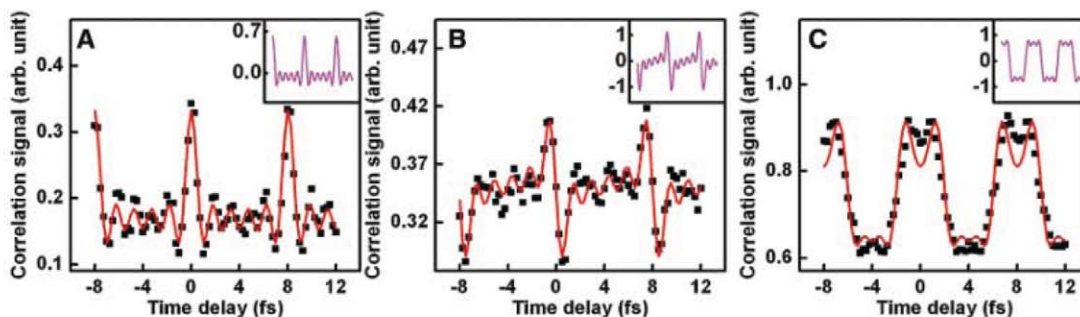


Fig. 4. Cross-correlation signals obtained for a few of the pulse trains shown in Fig. 1B. The amplitudes required for the syntheses are given in table S1: (A) transform-limited subcycle cosine (CEP = 0); (B) transform-limited subcycle sine (CEP = $\pi/2$); (C) square pulses with CEP = 0. The corresponding reconstructed fields are shown in the insets.



used to plot the solid curve in Fig. 2C, which shows good agreement between simulation and experimental data. At zero time delay, the combined $E_1(t)$ and $E_b(t)$ equals $E(t)$, the field of the synthesized sawtooth. This field can be Fourier reconstructed from the CEP and the amplitudes (Fig. 2D). A comparison of Fig. 2, C and D, indicates that the sawtooth field is nearly completely retrieved, thus confirming the efficacy of the shaper-assisted linear cross-correlation technique. Given the many sources that lead to amplitude fluctuations and phase distortions, this result demonstrates the robustness of the synthesized pulses, permitting successful field retrieval.

The instantaneous field of a Fourier-synthesized waveform is CEP dependent, whereas the pulse envelope is not [for an illustration, see figure 3 in (20)]. This result can now be experimentally validated here. Using the same sawtooth as an example, we recorded the $I(\tau)$ of waveforms that have identical field amplitudes but different CEPs. The results are shown in Fig. 3, where the waveforms have the same normalized amplitudes as those in Fig. 2 but three different values of the CEP ($\pi/2$, π , and $3\pi/2$). The effect of the CEP on the shape of the electric field as predicted by Fourier analysis is clearly displayed.

The procedure we have described can be extended to synthesize and retrieve various repetitive fields. For example, transform-limited subcycle sine and cosine pulse trains are formed with normalized amplitudes of $\{1, 1, 1, 1, 1\}$ for five harmonics and a CEP of $\pi/2$ and 0, respectively. A square wave has amplitudes of $\{1, 0, -1/3, 0, 1/5\}$ and a CEP of 0. Cross-correlation of these synthesized fields is shown in Fig. 4.

The average power of the beam of synthesized pulses in this experiment was a few milliwatts. This power is limited mainly by the power of the generated fifth harmonic and the repetition rate of the pump laser. One desirable improvement is to substantially increase the duty cycle of the laser. Raising the pump laser pulse energy and repetition rate, as well as using a larger beam size, could easily increase the average power to the watt level. Starting with a continuous-wave (CW) or quasi-CW laser source could make the synthesized waveforms inherently more stable. Several groups are making substantial progress in this direction (21, 22).

Despite the limited number of waveforms that can be synthesized with five harmonics, we have

described a simple solution to an optical waveform synthesizer that is analogous to RF and microwave function generators and showed that several common waveforms are accessible. We further showed an all-optical scheme to retrieve the instantaneous field. For broad application, it is desirable to have many more spectral components available for the synthesis. Other research groups have obtained >200 sidebands by driving the rotational Raman and vibrational Raman coherence simultaneously (23).

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Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1198397/DC1
Materials and Methods
Figs. S1 to S3
Table S1
Movie S1

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Layer-by-Layer Removal of Graphene for Device Patterning

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The patterning of graphene is useful in fabricating electronic devices, but existing methods do not allow control of the number of layers of graphene that are removed. We show that sputter-coating graphene and graphene-like materials with zinc and dissolving the latter with dilute acid removes one graphene layer and leaves the lower layers intact. The method works with the four different types of graphene and graphene-like materials: graphene oxide, chemically converted graphene, chemical vapor-deposited graphene, and micromechanically cleaved ("clear-tape") graphene. On the basis of our data, the top graphene layer is damaged by the sputtering process, and the acid treatment removes the damaged layer of carbon. When used with predesigned zinc patterns, this method can be viewed as lithography that etches the sample with single-atomic-layer resolution.

The electronic properties of graphene (I), a two-dimensional (2D) network of sp^2 carbon atoms, can vary as a function of the number of carbon layers in the sample (2, 3).

Existing methods for patterning graphene do not allow one to control the number of layers removed (4–7). Some of them are designed to work with a single existing layer of carbon material (4, 5),

whereas others that were applied toward few-layer graphene (FLG) indiscriminately etch all of the layers present in the sample down to the supporting substrate (6, 7).

In a typical single-atomic-layer lithography procedure (Fig. 1A), we coated graphene that was supported on a Si/SiO₂ wafer with 5 nm of zinc metal by sputtering (8). The sample was then placed in dilute HCl solution (typically 0.02 to 0.1 M) for 3 to 5 min to dissolve the zinc. Lastly, the sample was rinsed with water and dried in a stream of nitrogen. This series of steps removes one layer of carbon, and the procedure can be repeated to remove additional carbon layers. For patterning of FLG, zinc was deposited on the targeted areas only, thus removing a carbon layer strictly from those zinc-coated areas.

The method was used initially on graphene oxide (GO) and chemically converted graphene (CCG). The GO was prepared according to the Hummers method (9), modified as we described previously (8, 10). The effect of treatment of the same GO flake in the course of two consecutive treatments is shown in Fig. 1, B to D. The scanning electron microscopy (SEM) image of the original flake (Fig. 1B) is dark; the features are barely visible. The flake appears semitransparent after the first treatment (Fig. 1C) and almost completely transparent after the second treatment (Fig. 1D). The flake's wrinkles and folded areas, which survived after the second treatment (Fig. 1D), show the outline of the original flake.

A similar set of SEM images is shown for patterned GO samples (Fig. 1, E to G). The owl image on the GO flake that was electron-beam (e-beam)-patterned and Zn/HCl-treated (Fig. 1E) shows that one GO layer can be removed in one treatment. Figure 1, F and G, shows different fragments of a continuous few-layered GO film atop a Si/SiO₂ substrate. The samples were first exposed to one Zn/HCl treatment in the shape of horizontal stripes and then to one more treatment in the shape of vertical stripes, as shown in schematic Fig. 1A. The treated areas appear lighter in the SEM when compared with the untreated areas, and the GO film acquires a transparent appearance. The underlying flakes are not removed from the treated areas. The lower-layer flakes that extend from the treated areas through the untreated areas are visible. Note that even in the areas exposed to two treatments where horizontal and vertical stripes overlap (marked with "n-2" on Fig. 2, F and G), the number of remaining layers is not zero. The lower layers that remain are visible in the areas where the original film contained three and more GO layers.

In order to determine the number of carbon layers removed, we supplemented SEM images

with atomic force microscopy (AFM) analysis, which in this case is a sensitive tool because of the large interlayer distance in the GO samples. Figure 2 shows a GO flake during the course of two consecutive Zn/HCl treatments. The SEM image of the original flake (Fig. 2A) was dark, whereas the AFM image (Fig. 2B) was well pronounced. The thickness of the flake's main body (Fig. 2C) was 2.2 to 2.3 nm, which corresponds to two carbon layers of highly oxidized GO (11–13). After the first Zn/HCl treatment, the flake became semitransparent in the SEM image (Fig. 2D), and the wrinkles and folded areas were visible. The AFM image (Fig. 2E) indicated a smaller height difference. The thickness was 0.8 to 0.9 nm (Fig. 2F), which corresponds to one layer of reduced GO or, namely, CCG (13–15). After the second Zn/HCl treatment (Fig. 2, G and

H), the flake disappeared, and the wrinkles and folded areas were the only parts still observable. The height profiles (Fig. 2I) were flat, suggesting that no layered carbon material was left on the surface except at the former folded regions. The sharp peaks in the height profiles 5 and 6 are caused by particulate contamination. These SEM and AFM images along with the height profiles show that two layers of the bilayer GO flake are sequentially and controllably removed during two consecutive Zn/HCl treatments.

Next we applied the method toward chemical vapor-deposited (CVD) graphene (Fig. 3), which was grown on copper and transferred to a Si/SiO₂ substrate (16). Bilayer CVD graphene was fabricated by stacking two independently grown monolayer graphene films. As has been shown, Raman spectroscopy can be used as a sensitive tool to

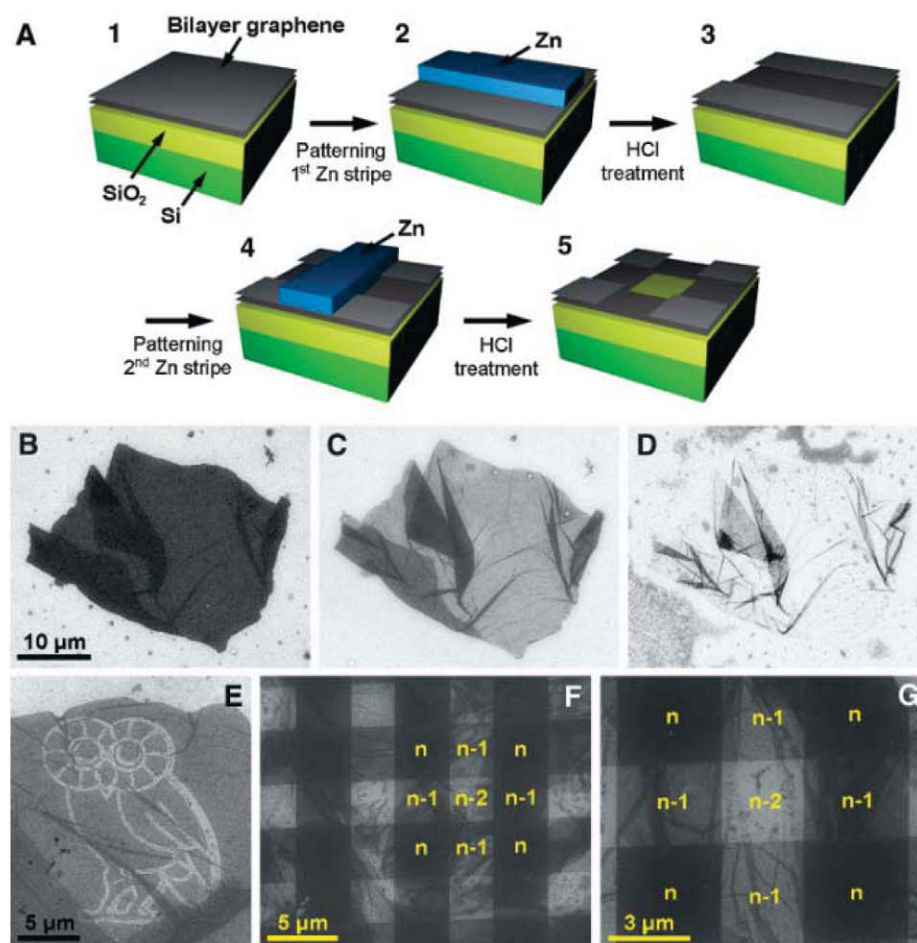


Fig. 1. Controlled layer-by-layer removal of graphene. (A) Schematic illustration of the method: (Step 1) The bilayer graphene on top of a Si/SiO₂ substrate. (Step 2) A patterned layer of zinc metal is sputtered atop the graphene. (Step 3) The zinc is removed by aqueous HCl (0.02 M) in 3 to 5 min with simultaneous removal of one graphene layer. (Step 4) Patterning of a second zinc stripe. (Step 5) HCl treatment removes the second stripe of zinc plus the underlying carbon layer. (B to D) SEM image of the same bilayer GO flake: (B) original, (C) after the first, and (D) after the second Zn/HCl treatment. (E) SEM image of a monolayer GO flake patterned in the image of an owl. (F and G) SEM images of a continuous GO film patterned with horizontal and vertical stripes in two consecutive Zn/HCl treatments. The lightest squares (an example is marked with "n-2", where the horizontal and vertical stripes overlap, represent areas exposed to two treatments. Areas exposed to one treatment (examples are marked with "n-1") are with a shade between the lightest and darkest squares. The darkest squares (examples are marked with "n") represent the areas with the original untreated GO film.

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determine the number of graphene layers (17, 18). Figure 3A is the Raman spectrum of the bilayer graphene film fabricated by stacking two monolayer sheets. The spectrum for the monolayer is taken from the areas where the two precursor sheets do not overlap, and thus it keeps its monolayer identity. The two spectra in Fig. 3A were normalized to bring the 2D peaks to the same height. The following changes occurred in the Raman spectrum as the second graphene layer was added: the G band dramatically increased in intensity and slightly red-shifted. A blue shift of 10 cm^{-1} was observed for the 2D band, with some increase of full width at half maximum value. These characteristics are indicative of the change in the electron bands caused by the interactions of the graphene layers (17, 18) and are consistent with the presence of misoriented bilayer graphene (19–22). The relative intensities of the G and 2D peaks are distinct for the two spectra and consistent with earlier reports (20–22).

The as-fabricated bilayer graphene film was patterned with horizontal stripes in one Zn/HCl treatment (Fig. 1B). In the areas where the top layer was selectively removed, the Raman spectrum shows the presence of monolayer graphene only. The increase in D-band intensity (Fig. 3C versus Fig. 3A bottom) indicates slight disruption of the remaining layer, or it might be caused by residual stripped carbon impurities.

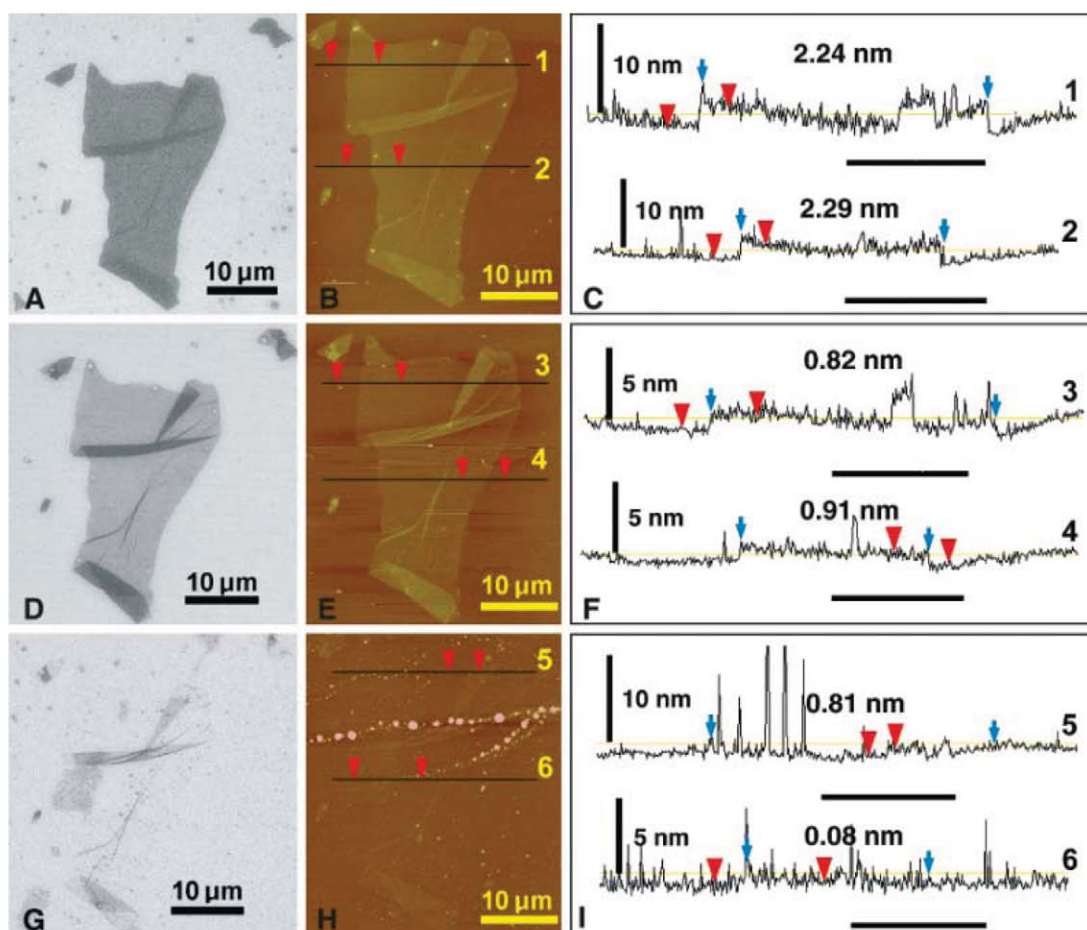
In addition to Raman spectral analysis, the electrical properties of devices fabricated on a bilayer CVD-graphene film were measured before and after the Zn/HCl treatment. The channel width was $3\text{ }\mu\text{m}$, and the channel length was $10\text{ }\mu\text{m}$. For 60 devices measured, the conductivity decreased 2 to 10 times after removing of one layer from an original bilayer device. The current-voltage [$I(V)$] curves before (black) and after the treatment (red) are shown in Fig. 3D for one of the devices where conductivity decreased by a factor of 6 after the treatment, which is an average for the series of experiments. The conductivity of bilayer graphene has been shown to be 4 to 10 times higher than that for monolayer graphene because of the interaction of the bottom layer with the SiO_2 substrate (13, 23). Moreover, the 2-to-10 range that we recorded is not likely to be associated with the reproducibility of the Zn/HCl treatment method but with inherent differences in the original graphene samples, such as mismatched interaction between graphene layers and corrugations between the layers. We find similar inconsistencies in electrical properties of as-grown monolayer CVD graphene itself, wherein devices fabricated on the same monolayer graphene film but separated by just $5\text{ }\mu\text{m}$ show differences in conductivities in this same range.

Finally, the graphene layer removal method was effective when applied to few-layer micro-

mechanically cleaved graphene and highly ordered pyrolytic graphite (HOPG). As shown in figs. S1 and S2, the Zn/HCl protocol can be used to sequentially remove layer after layer of the micro-mechanically cleaved graphene.

In order to probe the mechanism of the reaction, in a control experiment we deposited zinc metal on CVD graphene by thermal evaporation instead of sputtering. No change in the graphene structure was observed after treatment with HCl. However, a carbon layer was successfully removed from GO and CVD graphene by sputter-coating 5 nm of aluminum and dissolving the latter in either 0.1 M HCl or in concentrated aqueous NaOH. Thus, a key factor in removing the carbon layer in graphene is the sputtering process, which forms defects in the top layer, allowing its further removal. Sputtered metal atoms have about 100 times more energy than those produced by thermal evaporation (24). It has been shown that irradiation of graphite by low energy ion beams produces atomic-size defects that do not extend further than the top layer (25, 26). The threshold energy for carbon atom displacement has theoretical estimates of 18 to 22 eV and experimental values of 18 to 20 eV for e-beam irradiation (27). At the same time, the corresponding experimental energy values for ion irradiation are $>30\text{ eV}$, with the most frequently reported being 31 to 34.5 eV (28–30).

Fig. 2. Bilayer GO flake in the course of two consecutive treatments. (A) SEM image, (B) AFM image, and (C) height profiles of the original bilayer GO flake. (D to F) Corresponding images and height profiles for the same flake after the first Zn/HCl treatment. (G to I) Images and height profiles of the same flake after the second treatment. Height profiles 1 to 6 on (C), (F), and (I) are taken across the correspondingly numbered lines indicated on (B), (E), and (H). The blue arrows on the height profiles indicate the flake's boundaries, and the red triangles indicate the locations between which the heights were determined. All the horizontal scale bars indicate $10\text{ }\mu\text{m}$.



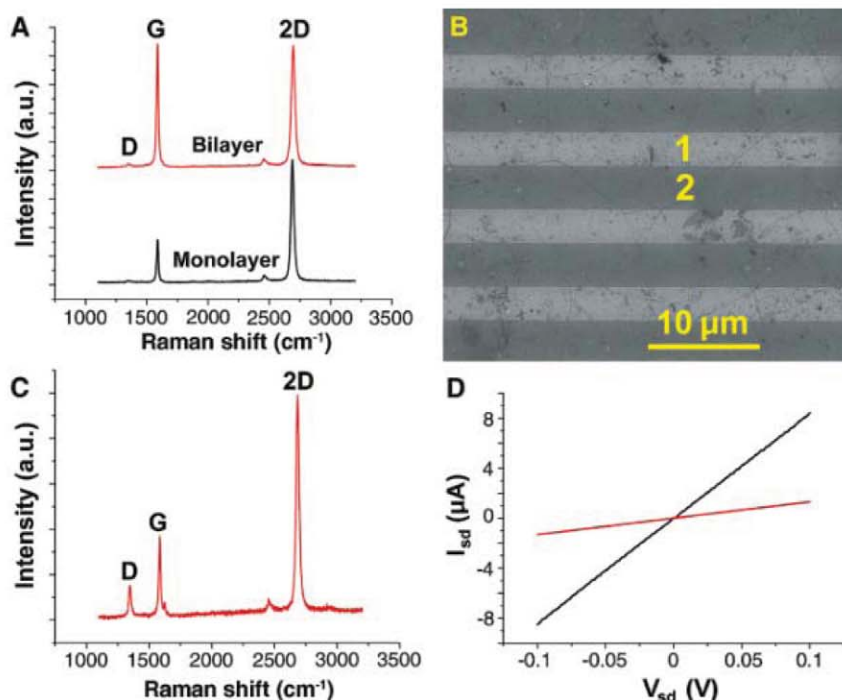


Fig. 3. Removing a carbon layer from CVD graphene. **(A)** Raman spectra of a bilayer CVD graphene film (top) as fabricated by stacking two monolayer (bottom spectrum) graphene films. The two spectra are scaled to have the same 2D peak height. a.u., arbitrary units. The Raman spectra here and in **(C)** were taken with 514-nm laser excitation. **(B)** SEM image of a bilayer CVD graphene film patterned by horizontal stripes in one Zn/HCl treatment. The yellow numbers indicate the number of carbon layers present after the treatment. **(C)** Raman spectra of the bilayer graphene film exposed to one Zn/HCl treatment [marked with "1" in **(B)**]. **(D)** Two I/V curves measured for the same device: the as-fabricated bilayer graphene (black line) and after exposing it to one Zn/HCl treatment (red line).

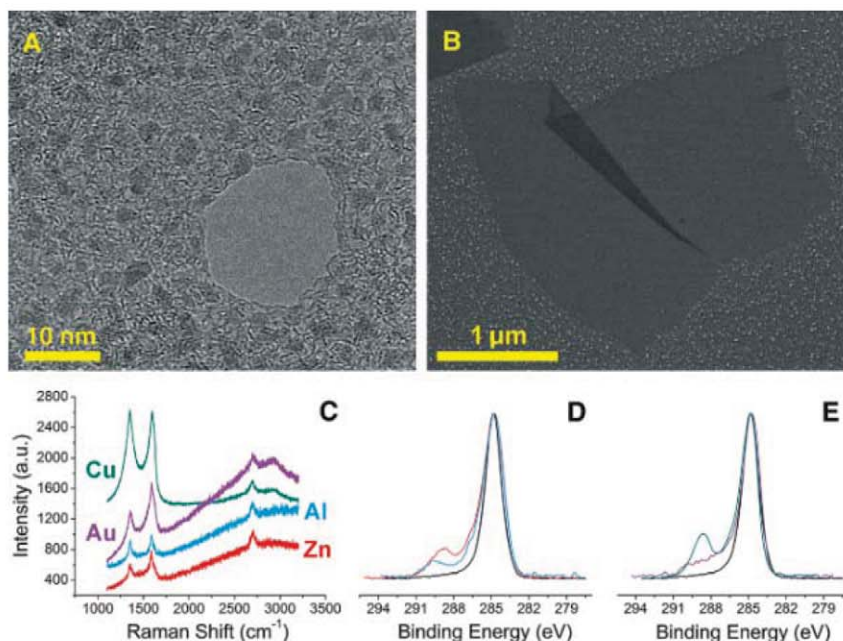


Fig. 4. Analysis of metal-sputtered graphene samples. **(A)** TEM image of monolayer CVD graphene sputter-coated with 0.6 nm zinc. Dark, 1- to 3-nm-sized zinc clusters are visible on the graphene surface. **(B)** SEM image of a GO flake on a Si/SiO₂ wafer sputter-coated with 0.6 nm zinc. Zinc clusters appear on the wafer surface, but no clustering is observed on the GO surface. **(C)** Raman spectra and **(D and E)** C1s XPS spectra of monolayer CVD graphene on a Si/SiO₂ wafer sputter-coated with 0.6 nm of four different metals: zinc, aluminum, gold, and copper. In **(D)**, black line, original graphene; blue, aluminum-coated; and red, zinc-coated. In **(E)** black line, original graphene; dark cyan, copper-coated; and magenta, gold-coated.

These values are ion dependent, and the values for Ar⁺ and Kr⁺ are 47.3 eV and 80.8 eV, respectively, to yield one-carbon atom vacancy defects (26). There are no reports of threshold energies for metal atoms needed to form similar defects. The average energy for sputtered metal atoms, including zinc, is 0.5 to 3 eV (24, 31). For zinc, the percentage of atoms having 20 eV and 34 eV is only 0.8% and 0.5%, respectively (31); hence only a small number of the sputtered atoms will have sufficient energy for carbon-atom displacement in the graphene. However, the ability to form defects is not solely a function of the incident particle energy, but it also depends on its chemical identity (26, 30).

To further investigate the effect of sputtering on graphene, we separately sputter-coated CVD graphene and GO samples with as little as 0.6 nm of zinc, aluminum, copper, or gold, followed by analysis of the sputtered products before dissolving the metals. Assuming a densely packed and uniform coating, 0.6 nm should provide a three- to four-atom-thick layer of metal atop of graphene. Figure 4A is a transmission electron microscopy (TEM) image of CVD graphene suspended on a grid after sputter-coating with 0.6 nm of zinc. Although sputtering forms some holes in the graphene, the larger part of the carbon network is not broken. The graphene sheet not only remained suspended on the grid but also supported the zinc clusters. However, the effect of sputtering onto graphene atop a SiO₂ surface might be different from graphene suspended on a grid. Zinc atoms sputtered atop GO samples on a Si/SiO₂ substrate under the same conditions do not form discernible clusters in the SEM (Fig. 4B). The zinc nanoparticles are visible on the Si/SiO₂ substrate surface but not on the GO flake. The zinc atoms likely form chemical bonds with the oxygen functionalities of the GO, thus preventing the agglomeration of the zinc.

Raman spectra of the sputter-coated CVD graphene samples (Fig. 4C) have the features of amorphous carbon (18, 19), suggesting that the graphene conjugation has been severely disrupted. The carbon 1s (C1s) x-ray photoelectron spectroscopy (XPS) data (Fig. 4, D and E) also exhibit noticeable changes in comparison with untreated graphene. The main carbon peak at 284.8 eV broadens, and a small carbonyl group signal appears at 288.0 to 289.0 eV. The main peak broadening is consistent with the presence of sp³-hybridized carbon in addition to sp²-hybridized carbon (32), suggesting a substantial transformation in the structure. The presence of carbonyl groups provides evidence for the formation of vacancy defects because carbonyl groups can exist only at the edges of graphene. The effect of different metals on the XPS spectrum is apparent. Gold and copper cause less main peak broadening, but they introduce oxygen functionalities, as evidenced by the shoulder in the 286.0 to 289.0 eV region for gold and the well-pronounced C=O peak at 288.6 eV for copper. Aluminum deposition leads to broadening of the main carbon peak,

as does zinc, but aluminum does not afford as many carbonyls. Lastly, zinc sputtering causes both the main peak to broaden and oxidizes graphene to produce carbonyl groups. The oxidation can be explained by reaction of the carbon radicals at the newly formed defect sites with traces of oxygen present in the sputtering chamber (5×10^{-5} torr) or upon subsequent exposure to air. The XPS data indicate that zinc sputtering, among the four tested metals, most affects the graphene as seen by the broadening of the main C1s signal. Interestingly, GO samples sputter-coated with metals behave somewhat differently than do CVD graphene samples, but both form similar materials characterized by defective carbon layers with a significant content of carbonyl groups; the results of those experiments are in (8) and fig. S3.

Sputtering damages graphene but does not remove it. Dissolving the zinc in acid is an essential step in removing the carbon layer. To investigate the role of the dissolving agent, we treated sputtered metals with different solutions. It was found that the carbon layer was removed when dissolving sputtered zinc using HCl or when dissolving sputtered aluminum using either HCl or concentrated NaOH. In all of those mixtures, H₂ gas is evolved. Conversely, no carbon layer was removed when dissolving sputtered copper in HCl/CuCl₂ or when dissolving sputtered gold in KI/I₂; no gas is evolved in those metal dissolutions. Although gas evolution probably plays a role in the carbon layer removal, a further difference of the two carbon-stripping metals, zinc and aluminum, versus the non-carbon-stripping metals, copper and gold, is in

their oxidation potentials. The combination of top-layer hole formation in the graphene, the large oxidation potentials of zinc and aluminum, as well as the evolved gas during their dissolution, all likely contribute to the mechanistic efficacy of the process.

The resolution that should be obtainable from this technique is being primarily dictated by the resolution of the metal patterning method. A 100-nm-wide zinc line was formed atop graphene with e-beam lithography, and a commensurately sized removal of the carbon layer was afforded by treatment with HCl (fig. S4). Furthermore, on the basis of AFM data, no delamination on the fabricated pattern corners or edges was observed while removing carbon layers from GO or HOPG. Over larger areas, scrolling of the edges was never observed by SEM.

We have demonstrated layer-by-layer removal of carbon from four types of graphene. This work demonstrates single-atomic-layer lithography in multilayer graphene structures and could provide impetus for the design of new graphene device embodiments.

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Figs. S1 to S4

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Helix-Rod Host-Guest Complexes with Shuttling Rates Much Faster than Disassembly

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Dynamic assembly is a powerful fabrication method of complex, functionally diverse molecular architectures, but its use in synthetic nanomachines has been hampered by the difficulty of avoiding reversible attachments that result in the premature breaking apart of loosely held moving parts. We show that molecular motion can be controlled in dynamically assembled systems through segregation of the disassembly process and internal translation to time scales that differ by four orders of magnitude. Helical molecular tapes were designed to slowly wind around rod-like guests and then to rapidly slide along them. The winding process requires helix unfolding and refolding, as well as a strict match between helix length and anchor points on the rods. This modular design and dynamic assembly open up promising capabilities in molecular machinery.

Controlling motion at the molecular scale is key to the engineering of synthetic nanomachines (1–7). Such control can be exerted upon introducing irreversible bonding between different moving parts of a molecular structure so that motion only takes place in defined directions. In rotaxanes (8–11), rings are irreversibly locked

around rods along which they may slide. In catenanes (12), interlocked rings may rotate around one another. An important improvement to these designs would entail not forbidding undesired motions but rather segregating them to much longer time scales, just as biological molecular machines self-assemble and disassemble slowly while their work

regime is rapid (13). We now have implemented this concept in molecular shuttles that slowly bind to rods and then rapidly slide along them without requiring an irreversible attachment. The shuttles consist of oligomeric aromatic amide molecular tapes that fold into very stable helices possessing a cylindrical cavity with high affinities for complementary rod-like guests. A guest terminated with bulky ends cannot thread itself in the cavity but nevertheless forms a complex via a slow helix unfolding-refolding process, thereby allowing the helix to rapidly shuttle along the guest without dissociating.

Aromatic oligoamides **1**, **2**, and **3** (Fig. 1A) were designed according to well-established rules (14, 15) to fold into helical structures stabilized by local preferential conformations at aryl-amide linkages and intramolecular π - π interactions between aromatic groups (fig. S1) (16). Based on studies of

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related compounds (17, 18), we predicted that the fluoroaromatic units in the center of the sequence would form a helix wide enough to accommodate an alkyl chain but nothing much larger, with each peripheral 2,6-pyridinedicarboxamide unit acting as a hydrogen bond donor to anchor the guest at a defined position in the helix cavity. Increasing strand length (from **3** to **1**) adjusts the distance between the anchor points along the helix axis and their relative orientation in a plane perpendicular to the helix axis. It also tunes the time scale of helix unfolding and refolding dynamics, which may range from fractions of a second to minutes, hours, and even days for multimeric helices, as demonstrated for helical quinoline-carboxamide oligomers (19).

Like other aromatic amide oligomers, compounds **1** to **3** fold into single-helical conformers, which then aggregate to form double-helical duplexes (Fig. 1C) (15, 16, 20). The structures of

the double helices (**2**)₂ and (**3**)₂ have been characterized in the solid state by x-ray crystallography (Fig. 2, A and B). Crystals of (**1**)₂ were also obtained, but their structure could not be solved due to very large unit cell parameters and poor diffraction intensity. In solution, the double helices prevail, and no signal of the single helices can be detected at equilibrium at 25°C by nuclear magnetic resonance (NMR) spectroscopy, even at low concentration. However, heating to 80°C in C₂D₂Cl₄ causes some duplex dissociation. The proportion of single helix is the same whether the sample is brought to 80°C by heating or by cooling, indicating that equilibrium is reached rapidly at this temperature (20). Under these conditions, integration of the single and double helix signals gives a measure of dimerization constants at 80°C: 2.8×10^5 , 6.4×10^5 , and $>10^6$ liter mol⁻¹ for **1**, **2**, and **3**, respectively.

Anticipating the need to match helix length with guest length, we prepared a range of rod-

shaped guests, **4a** to **4g**, incorporating a successive number of methylene units. We then treated each in turn with **1**, **2**, or **3** in CDCl₃ solutions and monitored changes by ¹H NMR (nuclear magnetic resonance). In some cases, the signals of the duplexes disappeared, and a new species emerged corresponding to a complex in which a single helix is wound around a rod-shaped guest (Fig. 1, C and D to G). Because the kinetics of host-guest complex formation can be extremely slow at room temperature (see below), all titrations were carried out at 45°C and binding constants calculated at that temperature (Fig. 1H). A guest with no stopper was prepared (**5**) to assess the mechanism of complex formation. Guests with 4,4-diphenyl-butyl stoppers (e.g., **6** and **7**) are more soluble and less prone to crystallize on their own than those with benzyl stoppers and proved to be more suitable for the crystallographic analysis of the complexes.

The stoichiometry and structure of the host-guest complexes were assessed in the solid state (Fig. 2), in solution, and in the gas phase. In solution, multiple intermolecular nuclear Overhauser effect (NOE) correlations were observed between aromatic amide protons of the helices and aliphatic alkyl protons of the guests (fig. S18); some of the guests' protons became diastereotopic in complexes due to the chiral environment of the helix (Fig. 1G); aromatic signals of the complexes are shifted downfield from those of the double helices, indicating weaker ring current effects and thus less extensive π -stacking as expected in the single helix (Fig. 1, D to G). Diffusion-ordered spectroscopy (21) showed that the diffusion coefficient of the complex is similar to (slightly higher than) that of the double helix (fig. S19). Both titration data in solution and mass spectrometry in gas phase confirmed that the complexes consist of a monomeric helix and one equivalent of guest (fig. S24). Crystallographic data confirmed the structure in the solid state and showed that the 2,6-pyridinedicarboxamide units of the helices hydrogen bond to the carbonyl groups of the guests (fig. S25). It follows that the complexes form only when the helix and the guest match in length with a tolerance of at most one CH₂ unit of the guest (Fig. 1H). Helices longer than **1** are expected to bind selectively to longer guests, demonstrating a high modularity in space of this system. The affinity of the single helices for their guests is clearly high enough to overcome their strong propensity to form double helices. This is facilitated by the fact that it takes two strands to form a duplex and only one to form a host-guest complex: The helix dimerization constant is in balance with the square of the rod-helix association constant.

The host-guest complexes between the shortest oligomers **2** and **3** and their respective guests form readily at 25°C. However, complexes involving **1** do not form even after days unless the mixture undergoes a heating-cooling cycle. The reasons lie in slow kinetics of complex formation from the single helix and in very slow kinetics of double helix dissociation into single helices (20). The pure single-helical conformer of **1** could be fortuitously

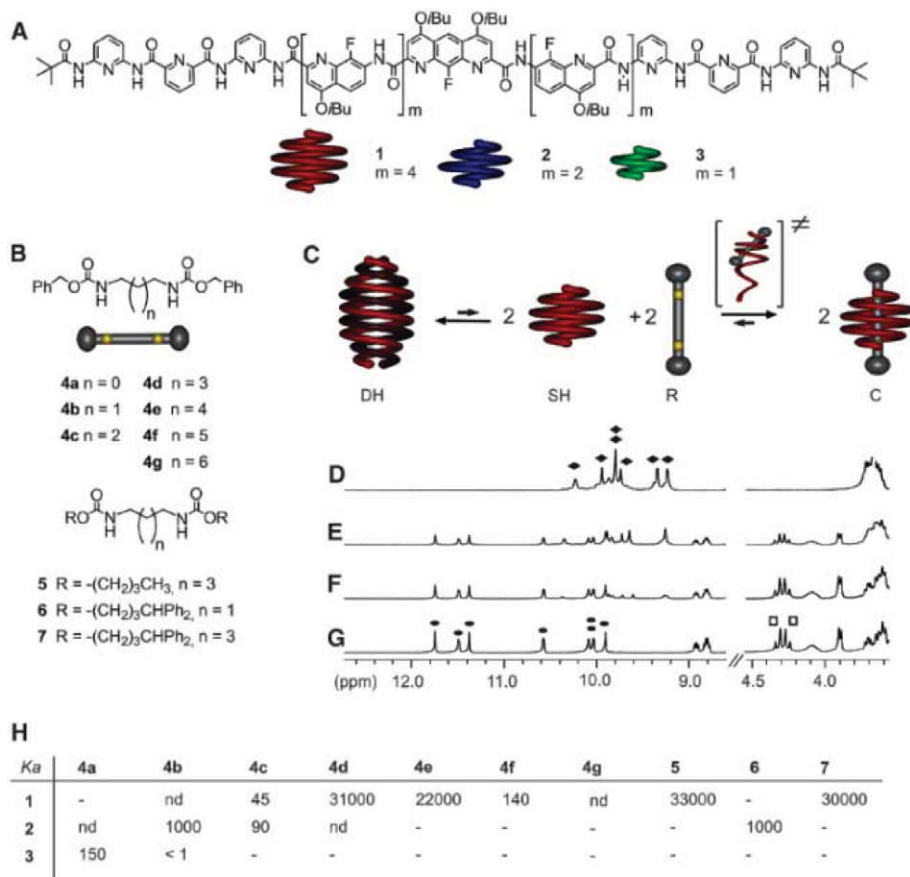


Fig. 1. Host-guest components and assembly. (A) Formulas of **1**, **2**, and **3**. Single-helical conformations span 4.5, 3.5, and 2.5 turns, respectively. (B) Rod-like guests possessing carbonyl hydrogen bond acceptors (yellow dots) with bulky end groups (**4**, **6**, **7**) or without (**5**). Depending on length (*n*), each guest matches best with **1**, **2**, or **3** as a host. (C) Equilibrium between oligomers **1**, **2**, and **3** as double helices (DH), single helices (SH), or complexes (C) with rod-like guests (R). The formation of a complex with a dumbbell guest requires the unfolding and refolding of the helix. (D to G) Titration of **1** (2 mM) by **4d** [from (D) to (G): 0, 0.5, 1, 1.5 equiv. of **4d**] monitored by 400-MHz ¹H NMR in CDCl₃ at 45°C. The high temperature reduces the time needed to reach equilibrium after each addition of **4d**. The signals of the starting double helix (**1**)₂ (♦) progressively disappear while the signals of **1**•**4d** emerge (●). Benzylic protons of the bound **4d** appear as a diastereotopic pattern (□), which reflects the chiral environment of the surrounding helix. (H) Constants of formation at 45°C of the complexes between **1** to **3** and **4** to **7** defined as $K_a = [C]^2/([DH].[R]^2)$. nd: no binding detected.

isolated by selective precipitation from methanol even though it constitutes a minor component in solution. Upon dissolving it again in CDCl_3 , the double helix slowly forms over 24 hours at 2 mM if no guest is present (fig. S4). However, in the

presence of **7**, which possesses very bulky end groups, the complex **1**⋯**7** forms at 25°C over the course of 30 min, which allowed us to estimate a kinetic constant of dissociation slower than 2 day^{-1} (fig. S21). In contrast, **5** does not possess any bulky

group and forms a complex **1**⋯**5** at rates too fast to monitor. These results indicate that complexes such as **1**⋯**5** may form by a simple threading of the guest into the helix cavity, whereas the formation of **1**⋯**7** requires an unwinding and rewinding of the helix around the guest (Fig. 1C) (22–24). The crystal structures of **1**⋯**7** and **1**⋯**5** shown in Fig. 2, D and E, illustrate that **5** is thin enough to thread itself into the helix of **1** whereas **7** is much too bulky to do so. These complexes relate to some structurally labile rotaxane structures in which molecular rings are closed by noncovalent bonds (25, 26) or by reversible covalent attachments (27, 28).

High host-guest stability and slow formation kinetics in the case of **1**⋯guest hint at even slower kinetics of complex dissociation. Indeed, complexes such as **1**⋯**7** can be eluted on a silica thin-layer chromatography plate without any observable dissociation (fig. S22). We thus endeavored to explore the extent to which helices of **1** wrapped around guests could undergo sliding motions more rapidly than they dissociate. Rod-like guest **8** possesses three hydrogen bond acceptors and thus two degenerate stations, which, because of their proximity, can bind only one helix at a time (Fig. 3A). Both solution and solid-state (Fig. 2F) data confirm that **8** forms a 1:1 complex with oligomer **1**. The rate of formation of **1**⋯**8** is almost identical to that of **1**⋯**7** (figs. S20 and S21). The single helix of **1** is C_2 symmetrical, but this symmetry is broken in **1**⋯**8** because one extremity of **1** lies near the central urea function of **8** whereas the other extremity is bound to one of the two carbamate functions. This loss of symmetry is seen in the ^1H and ^{19}F NMR spectra of **1**⋯**8**, which feature twice as many signals as symmetrical complexes. The two extremities of **1** should exchange their environments when **1** goes from one binding station of **8** to the other (Fig. 3B). Exchange spectroscopy NMR (29) experiments were carried out on solutions of **1**⋯**8** and showed clear correlation resulting from the motion of **1** between the two stations of **8**. This motion can be calculated to take place at rates between 2 and 4 min^{-1} at 25°C, a time scale considerably smaller than that of host-guest complex dissociation, implying that **1** exchanges between the two stations of **8** via a sliding (shuttling) process and not via a dissociation (unfolding)–association (refolding) mechanism.

A step beyond random sliding consists in triggering motion so that all molecules in an ensemble undergo the same change within a given time window. To reach this objective, we designed guest **9** with two different binding stations: one with a heptyl segment, the other with a diethylamine segment, with the expectation that a host-guest complex would form on the neutral amine segment but not on the corresponding ammonium. As with **8**, only a 1:1 complex forms between **1** and **9**, as confirmed by integrating the ^1H NMR signals belonging to **1** and to **9** in **1**⋯**9**. However, **1** may not reside equally at the two different stations of **9**. Indeed, ^1H and ^{19}F NMR spectra of **1**⋯**9** feature two sets of signals in slightly different

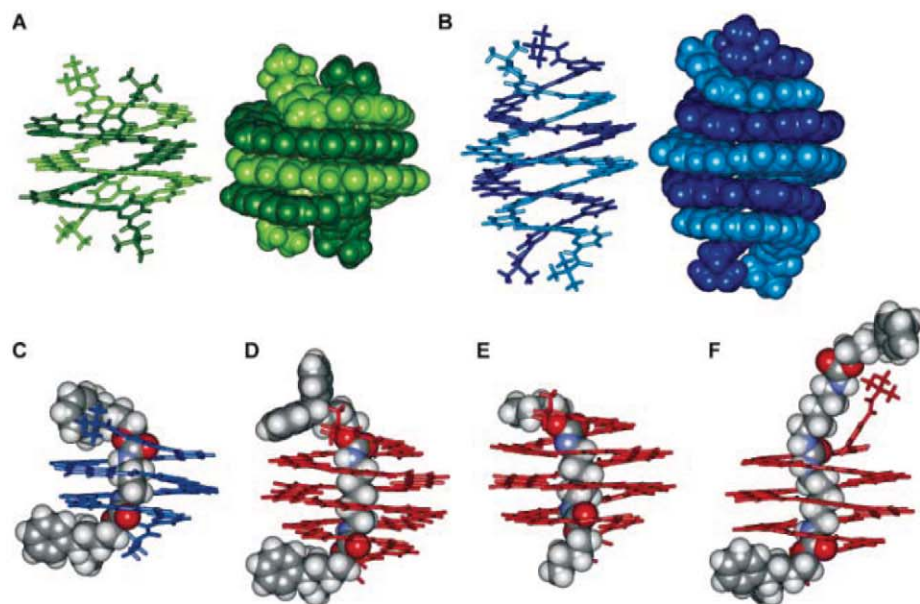


Fig. 2. Solid-state structures. (A) Double helix $(\mathbf{3})_2$. (B) Double helix $(\mathbf{2})_2$. (C) Complex **2**⋯**6**. (D) Complex **1**⋯**7**. (E) Complex **1**⋯**5**. (F) Complex **1**⋯**8**. Side chains (O-isobutyl groups) and included solvent molecules have been removed for clarity.

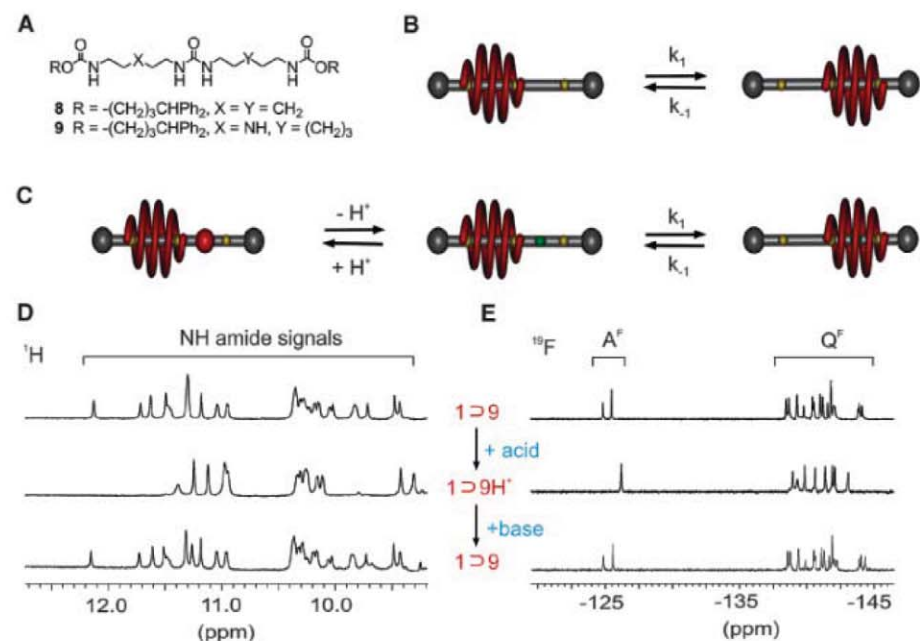


Fig. 3. Helix shuttling. (A) Formulas of rod-like guests having two adjacent identical (**8**) or different (**9**) stations. Because of the proximity between the two stations and the presence of a single carbonyl group in the middle of the rod, only one station at a time can be occupied by a helix. (B) Scheme of degenerate helix sliding along a symmetrical guest. The yellow dots mark the hydrogen bond acceptors. (C) Scheme of helix sliding along a nondegenerate guest possessing a station that can be blocked or unblocked upon protonation or deprotonation, respectively. The green dot symbolizes the amine function of **9**, which can be included in the helix cavity. The red dot is the corresponding ammonium, which is not included in the helix cavity. (D and E) Portions of the ^1H and ^{19}F NMR spectra of **1**⋯**9** in CDCl_3 at equilibrium, after adding excess of an organic acid (2,4-dinitrophenol) and after neutralizing with a base (Et_3N). A^{F} and Q^{F} designate fluoro-anthracene and fluoro-quinoline, respectively.

(58/42) proportions corresponding to a slight bias in favor of one station. Upon titrating **1** with an acid, the smaller NMR signals disappear without any measurable delay during the measurement of a ^1H NMR spectrum (~ 2 min), and only one set of signals remains after adding excess acid (Fig. 3, D and E). This is consistent with the trapping of **1** on a single station of **9**, as it is repelled by the ammonium function of the other station. Adding a base instantly reverses the process. Again, the time scale of this controlled motion is much faster than the rates of unfolding and refolding of **1** around **9**, implying that motion is mediated by the rapid sliding of **1** along **9**.

Using helices longer than **1** should expectedly result in slower sliding but also in much slower helix-rod dissociation. Combining rods with multiple distinct stations with mixtures of helices of different lengths should thus allow several controlled motions to proceed at different rates within a single supramolecular construct.

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Figs. S1 to S31

Tables S1 to S9

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Oxygen Isotope Variations at the Margin of a CAI Records Circulation Within the Solar Nebula

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Micrometer-scale analyses of a calcium-, aluminum-rich inclusion (CAI) and the characteristic mineral bands mantling the CAI reveal that the outer parts of this primitive object have a large range of oxygen isotope compositions. The variations are systematic; the relative abundance of ^{16}O first decreases toward the CAI margin, approaching a planetary-like isotopic composition, then shifts to extremely ^{16}O -rich compositions through the surrounding rim. The variability implies that CAIs probably formed from several oxygen reservoirs. The observations support early and short-lived fluctuations of the environment in which CAIs formed, either because of transport of the CAIs themselves to distinct regions of the solar nebula or because of varying gas composition near the proto-Sun.

Calcium-, aluminum-rich inclusions (CAIs) are understood to have formed very early in the evolution of the solar system and in contact with nebular gas, either as solid condensates or as molten droplets. In general, CAIs are ^{16}O -rich relative to planetary materials and are believed to record the $\Delta^{17}\text{O}$ (I) composition of solar nebular gas in which they grew (2). $\Delta^{17}\text{O}$ may be a marker of radial position within the solar nebula. Less prim-

itive nebular materials (such as iron-, magnesium-rich chondrules) typically have planetary-like values ($\Delta^{17}\text{O} = 0$) and may have formed further out in the protoplanetary disk from where CAIs formed (3). Previous oxygen isotopic studies document substantial variation in the $\Delta^{17}\text{O}$ of CAIs (2, 4), but because of their lower spatial resolution ($\geq 10\ \mu\text{m}$) have not been able to probe the isotopic stratigraphy of the outer parts of CAIs with enough resolution to detect the continuous range of isotopic variations observed here. Because models suggest that radial transport of primitive matter may have played an important role in the evolution of protoplanetary disks (5–7), evidence within individual CAIs for transfer among distinct regions in the solar nebula, such as systematic $\Delta^{17}\text{O}$ variations, is of critical importance.

To further investigate intra-CAI oxygen isotopic variations, a component of the CV3 carbonaceous chondrite Allende (the CAI called A37), its surrounding concentric rim, and a micro-CAI enclosed within this rim were measured with NanoSIMS, an ion microprobe with nanometer-scale spatial resolution. Measurements were obtained as $\sim 2\text{-}\mu\text{m}$ spot analyses spaced every 7 to $10\ \mu\text{m}$ across the rim and the outer $\sim 150\ \mu\text{m}$ of the interior (Fig. 1) (8). At the resolution that is accessible with NanoSIMS, both A37 and its rim exhibit more than 20 per mil (‰) variation in $\Delta^{17}\text{O}$, a range that is close to the full range thought to exist among solids formed in the solar system. Mass-dependent physicochemical processes cannot produce variations in $\Delta^{17}\text{O}$. These data imply that A37 was transported among several different nebular oxygen isotopic reservoirs (4), potentially as it passed through and/or into various regions of the protoplanetary disk.

Concentric multi-mineralic rim sequences [so-called Wark-Lovering (WL) rims (9)] are a widespread feature that indicates that many CAIs shared a similar evolution history to each other and possibly to less primitive materials, despite the compositional and mineralogical diversity of their interiors. These ubiquitous WL rims formed late—although still relatively early in solar system history according to evidence that they initially contained a canonical abundance of the short-lived nuclide ^{26}Al (10). The preservation of their primitive age attests to the fact that they have experienced minimal subsequent disturbance either in the nebula or on the chondrite parent body. The mineralogy and composition of the WL rims surrounding CAIs suggest that late in their evolution, the CAIs were in a nebular environment distinct from that where they origi-

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nated and closer in composition to the environment in which the building materials of the terrestrial planets formed (10).

The CAI studied here (A37) is a ~7- by 4-mm compact Type A inclusion. A37 is composed primarily of the minerals melilite [Ak_{20-40} (11)], 20- to 70- μm -sized spinel, anhedral perovskite and rare fassaite that mainly occurs between the melilite grains (Fig. 1) (12). The surrounding WL rim is ~50 to 100 μm thick and is made up of a typical layered mineral sequence. Both the interior and WL rim data exhibit large variations in $\Delta^{17}\text{O}$ (Figs. 2 and 3). Heterogeneity in $\Delta^{17}\text{O}$ has been explained by isotopic mixing between an ^{16}O -rich reservoir composed of refractory materials and a second reservoir (probably nebular gas) with a more “planetary-like” isotopic composition (13). On an oxygen three-isotope plot, the data for A37 scatter about and along the carbonaceous chondrite anhydrous mineral (CCAM) line (Fig. 2). Linear regression of the CAI interior data and WL rim data yields slopes of 0.89 ± 0.06 (2σ) and 0.96 ± 0.05 (2σ), respectively. A majority of the spinel $\pm$ hibonite spots in the WL rim have $\Delta^{17}\text{O}$ values less than -20‰ , which is comparable with spinel in the interior of other Allende CAIs (14) and equal to or lower than the melilite in the interior of A37, spinel in the micro-CAI, and the composition of many other unequilibrated inclusions (3).

The oxygen isotope zoning, from ^{16}O -rich values ($\Delta^{17}\text{O} \approx -20\text{‰}$) in the interior to near planetary-like values ($=0\text{‰}$) at the edge (Fig. 3), cannot be explained by igneous processes and is mostly likely secondary in origin. The inferred pre-rim history therefore involves the CAI first solidifying with a uniform enrichment of ^{16}O and then partially exchanging its oxygen with a second reservoir, most likely a nebular gas of planetary isotopic composition (15, 16). The detailed melilite oxygen-zoning profiles (Fig. 3) (8) provide support for this model in that they exhibit the same range of $\Delta^{17}\text{O}$ and nearly identical $\Delta^{17}\text{O}$ boundary-layer thicknesses.

We modeled solid-gas exchange of oxygen isotopes in A37 to investigate the possibility that the $\Delta^{17}\text{O}$ isotope profiles developed after crystallization and after the CAI was transferred from its place of origin to a distinct gaseous nebular reservoir (Fig. 4). For this, we obtained numerical solutions to the time-dependent diffusion equation in radial coordinates. Oxygen was assumed to diffuse within the solid in response to a change in the isotopic composition of oxygen in surrounding gas. The equation used is

$$\frac{\partial C_{i,\text{cond}}}{\partial t} = D_i \left(\frac{\partial C_{i,\text{cond}}}{\partial r^2} + \frac{2}{r} \frac{\partial C_{i,\text{cond}}}{\partial r} \right) \quad (1)$$

where $C_{i,\text{cond}}$ is the concentration of species i in the inclusion, D_i is the diffusivity of the isotope of interest in the inclusion (16), r is the radius of the inclusion, and t is time. The zoning in the model comes from solving the diffusion equation subject to constant concentrations of ^{16}O , ^{17}O ,

and ^{18}O at the gas-solid interface and assigning a uniform low- $\Delta^{17}\text{O}$ initial value to the CAI interior. The boundary condition at the CAI surface corresponds to a constant partial pressure of oxygen with a fixed (planetary-like) $\Delta^{17}\text{O}$ isotopic composition. The planetary $\Delta^{17}\text{O}$ value of the low-sodium melilite at the interface with hibonite/spinel, which is believed to be related to growth of the WL rims (fig. S2), is used to define the near-zero $\Delta^{17}\text{O}$ value at the hypothesized gas-CAI boundary. The $\Delta^{17}\text{O}$ value common to the CAI interior is used to estimate the enriched abundance of ^{16}O of the CAI before exchange with gas. The oxygen concentration of the gas is

based on a solar bulk composition at 10^{-3} bar total pressure (17, 18), and the concentration of oxygen in melilite is estimated by stoichiometry from electron probe analyses (8).

The systematic ^{16}O depletion in the outer margin of A37 can be fit with solid-state diffusional relaxation after an instantaneous change in the isotopic composition of the gas surrounding the CAIs (Fig. 4). Oxygen isotope exchange through self-diffusion is inferred to be the dominant mechanism in the development of the isotopic zoning profiles; the process is one involving only exchange of oxygen atoms between the CAI and the gas, with no other coupled chemical diffu-

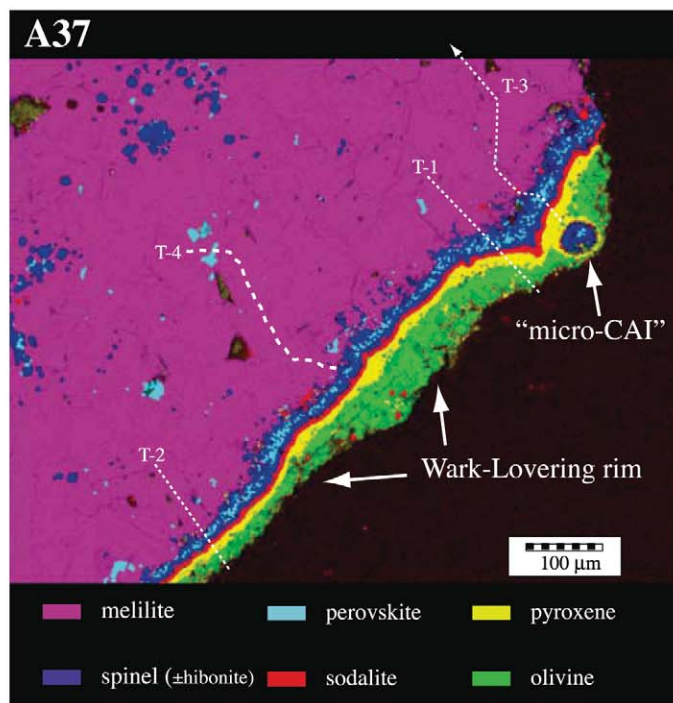


Fig. 1. Compositional x-ray image of the rim and margin of A37, a typical Type A CAI within the Allende meteorite. Oxygen isotope data were measured along traverses 1, 2, and 3 (by NanoSIMS), and compositional data were obtained from traverse 4 (by electron microprobe).

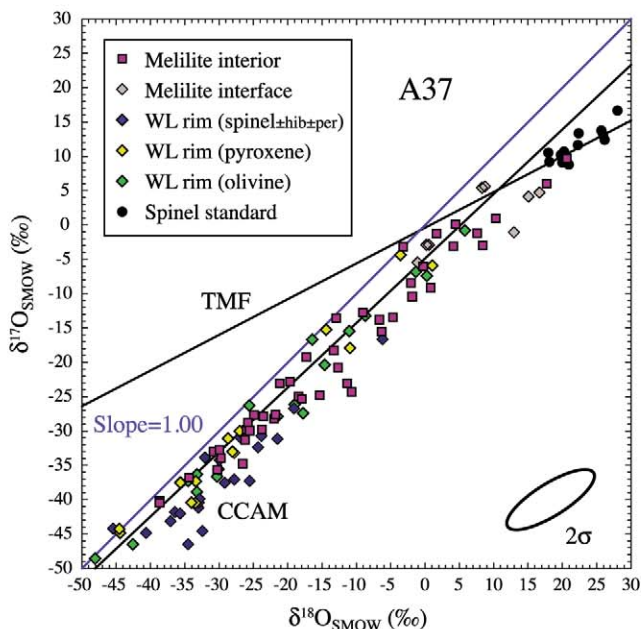


Fig. 2. Oxygen isotopic composition of refractory inclusion (A37) and rim. Data fall along the slope ~ 0.94 CCAM line. Terrestrial mass fractionation (TMF) line (slope = 0.52) and primordial mixing line (slope = 1.00) are shown for reference. Error ellipse represents 2σ external reproducibility of measurements.

sion. The time necessary to develop the radial ^{16}O depletions represented by traverses 1, 2, and 3 depends on the assumed temperature because of

the strong dependence of D_0 on temperature. The time scale is ~ 150 years at temperatures near the liquidus (~ 1700 K), but much longer ($\sim 530,000$

Fig. 3. Oxygen isotope zoning across the WL rim and outer margin typical of A37, defined by ion microprobe traverses. Black and white scale bars, $10\ \mu\text{m}$ per increment. Horizontal band $\Delta^{17}\text{O} = -15$ to -20 is representative of interior. Colors correspond to different phases as in Fig. 1.

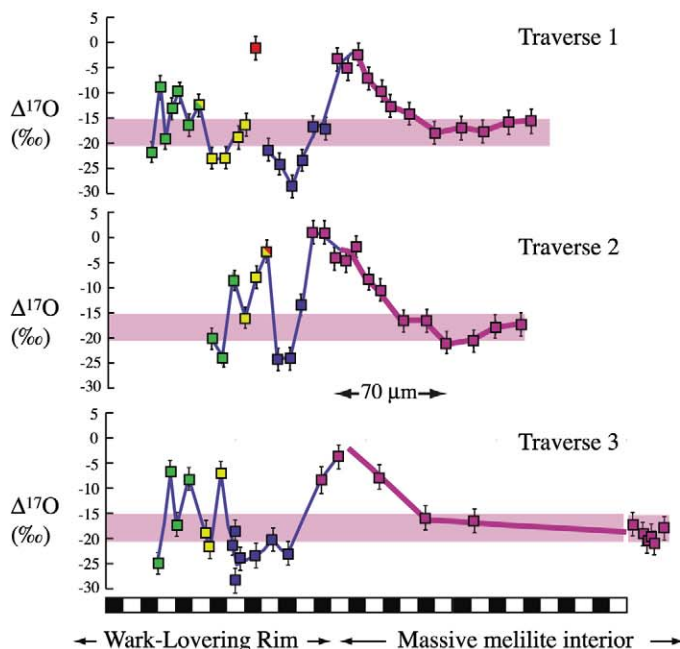
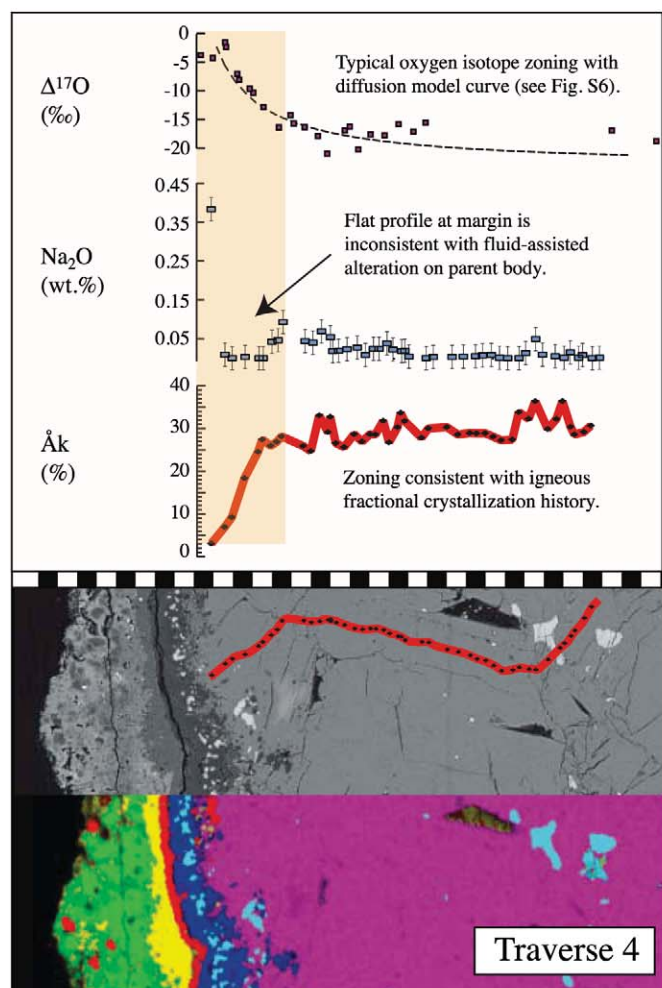


Fig. 4. Oxygen self-diffusion model compared with the isotope zoning of A37. Electron microprobe data show representative compositional [Åk (11) and sodium] zoning profiles (traverse 4) across the outer margin of melilite. Typical errors are shown for Na_2O . Black and white scale bars, $10\ \mu\text{m}$ per increment. Backscattered electron imaging (gray scale) and x-ray mapping (mineral color-coding as in other figures) accompany the electron probe data.



years) at 1200 K. Calculations (16) place an upper limit of 1600 K (and thus a lower limit of ~ 500 years) for the integrated reaction period of diffusive exchange on the basis of the fact that spinels within the interior of CAIs are commonly ^{16}O -rich (have exchanged $<5\%$ of their original ^{16}O -excesses) despite their small size ($\sim 50\ \mu\text{m}$). Isotopic exchange could have occurred by reheating in numerous short (hours to days) events (19)—for example, as the result of time (10^2 to 10^3 years) within ^{16}O -poor gas subjected to shockwaves (20), which is similar to the environment envisioned for chondrule formation (21, 22). Temperatures of ≤ 1000 K, the maximum obtained within undifferentiated planetesimals, require unreasonable heating times (>100 million years) to fit the data, given both the thermal histories of chondrite parent bodies (23) and the primitive age of the surrounding WL rims (10). The flat sodium profile across the outer margin of the melilite interior (Fig. 4) is inconsistent with transport of sodium inward after the rim formed. Thus, there is no evidence that the oxygen isotope profiles are related to fluid-assisted alteration processes on the chondrite parent body (14).

A simple model in which CAI minerals exchange with a planetary-like oxygen reservoir (15, 16) would suffice if the WL rims had uniform oxygen isotopic compositions similar to that of the CAI margin—that is, close to the terrestrial planets. This scenario is complicated by the ^{16}O -enriched composition of most of the WL rim mineral bands. Because the WL rim data scatter nearly continuously along the CCAM line, as the rim grew A37 probably experienced isotopic exchange with a planetary-like gas reservoir that was not on the primordial slope = 1.00 mixing line. Detailed textural and mineralogical investigations at the CAI-rim interface reveal a ~ 1 - to $5\text{-}\mu\text{m}$ -thick, discontinuous zone of melilite between the gehlenitic interior and the spinel $\pm$ hibonite of the rim that is åkermanite -rich and relatively sodium-poor (fig. S2). This relatively ^{16}O -poor zone may reflect new growth of magnesium-rich melilite or metamorphism of existing melilite that occurred concurrently with oxygen exchange and seems to mark a transition between growth from ^{16}O -rich to growth from ^{16}O -poor gas. This melilite possibly reflects the beginning of WL rim formation and implies that, initially, the rim grew from a ^{16}O -poor, planetary-like reservoir.

The transition from the åkermanite -rich melilite layer to the spinel $\pm$ hibonite layer (a distance of no more than $10\ \mu\text{m}$) records the change in oxygen isotope composition from planetary-like back to the most ^{16}O -enriched reservoir recognized in the solar system. Conceivably, the CAI and WL rim could have formed from a ^{16}O -rich gas followed by immersion in a ^{16}O -poor one. However, the large variability in $\Delta^{17}\text{O}$, specifically the inward-increasing trends within spinel (and other rim minerals), implies that late-stage mineral-specific, diffusion-driven oxygen isotope exchange with a single external reservoir is unlikely to explain the rim record (4). The pyroxene and olivine rim data within each traverse show

some individual and some shared zoning behavior (for example, early pyroxene appears to have grown from a more planetary-like gas) but in general imply growth from relatively ^{16}O -rich gas. Varying isotopic composition in the pyroxene layer is consistent with the range of $\text{Ti}^{3+}/\text{Ti}^{4+}$ ratios reported for rim pyroxene (10, 24), possibly reflecting formation in more ^{16}O -poor (likely oxidizing) and ^{16}O -rich (likely reducing) environments (10). Given the large variations in $\Delta^{17}\text{O}$ that exist within the WL rim and the outer margin of the melilite interior of A37, the data require exposure of the inclusion to several (at least two) distinct nebular oxygen reservoirs in addition to the one from which it formed. We believe that the inclusion condensed from an ^{16}O -rich gas and was subsequently exposed to ^{16}O -poor and then ^{16}O -rich reservoirs. Collectively, this isotopic and petrologic record provides our best account of the transfer of CAIs among distinct nebular settings within the protoplanetary disk.

Young protoplanetary disks evolve through viscous accretion to the star coupled with outward transport of angular momentum. The evidence reported here supports expectations that radial transport of solid matter—perhaps in both directions—is a basic consequence of protoplanetary disk evolution (5, 25, 26). Large-scale radial circulation of nebular solids is also consistent with the reports of crystalline material located in the outer reaches of our solar system (27, 28) and in the outer, cool regions of distant stars (29, 30). The variable but largely ^{16}O -rich

composition of the WL rim suggests that after transport out of the inner solar system, CAIs either continued to form within a region in the outer solar system that varied in composition or that they were returned back to the inner solar system. Whether CAIs shared any common history with other nebular materials, such as early forming chondrules, is uncertain.

References and Notes

- $\Delta^{17}\text{O}$ reflects the deviation of the oxygen isotopic composition from the terrestrial fractionation line, where $\Delta^{17}\text{O} = \delta^{17}\text{O} - 0.52\delta^{18}\text{O}$. $\delta^{17}\text{O}$ and $\delta^{18}\text{O}$ reflect the per mil difference from the composition of standard mean ocean water (SMOW) so that $\delta^{17}\text{O}_{\text{SMOW}} = 10^3[(^{17}\text{O}/^{16}\text{O})/(^{17}\text{O}/^{16}\text{O})_{\text{SMOW}} - 1]$ and where i refers to either 17 or 18.
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Supporting Online Material

www.sciencemag.org/cgi/content/full/331/6021/1175/DC1

Materials and Methods

Figs. S1 to S5

Tables S1 to S3

References

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Dietary Change and Evolution of Horses in North America

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The evolution of high-crowned molars among horses (Family Equidae) is thought to be an adaptation for abrasive diets associated with the spread of grasslands. The sharpness and relief of the worn cusp apices of teeth (mesowear) are a measure of dietary abrasion. We collected mesowear data for North American Equidae for the past 55.5 million years to test the association of molar height and dietary abrasion. Mesowear trends in horses are reflective of global cooling and associated vegetation changes. There is a strong correlation between mesowear and crown height in horses; however, most horse paleopopulations had highly variable amounts of dietary abrasion, suggesting that selective pressures for crown height may have been weak much of the time. However, instances of higher abrasion were observed in some paleopopulations, suggesting intervals of stronger selection for the evolution of dentitions, including the early Miocene shortly before the first appearance of Equinae, the horse subfamily in which high-crowned dentitions evolved.

The evolution of high-crowned molars (hypsodonty) in horses and other mammalian herbivores is a classic hypothesis of adaptation (1–3), thought to have evolved as a response to increased dental wear associated with changes in habitat structure that caused a higher degree of dietary abrasion, such as the spread of phytolith-bearing grasslands and the increased consumption of dust in increasingly arid environments (4–10). The earliest horses

from ~55.5 million years ago (Ma) had short-crowned (brachydont) molars with poorly developed shearing crests, suggesting a frugivorous diet. During the Eocene and Oligocene, horses acquired shearing lophs on their molars, suggesting a shift toward leafy browsing. The subfamily Equinae, which includes living horses, appeared in the early Miocene (~18 Ma). These horses show increased crown height, increased occlusal surface complexity, and thickened cementum. The appear-

ance of Equinae suggests a shift toward grazing (grass-eating) diets; however, paleosols and fossil phytolith assemblages suggest that grassy habitats were present in the North American Great Plains millions of years earlier (11–14). The earliest Equinae had intermediate crown heights (mesodont), whereas high-crowned (hypsodont) horses appeared millions of years later. Explanations for the delayed evolution of hypsodonty are that selection was weak or episodic, or that phylogenetic constraints caused horses to resist feeding in open grasslands for several million years (13).

To further understand the coevolution of horses and their paleodiets, we examined mesowear patterns in the molars of North American fossil horses from their first appearance (~55.5 Ma) to their extinction in North America (~0.01 Ma) (15). Their geographic coverage includes nearly all of the fossiliferous regions of North America; the greatest concentration of data is from the Great Plains. Mesowear is a macroscopic dietary

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proxy in which worn molar cusps are characterized by their sharpness and relief (16–19). Mesowear reflects the amounts of attritive (tooth-on-tooth) wear and abrasive (food-on-tooth) wear. Species that feed on leafy browse generally maintain sharpened, high-relief cusp apices and have low dental wear rates and low mesowear

scores (Fig. 1D). Grazers have cusp apices that become blunted from abrasive phytolith-bearing grasses and more grit encountered when feeding near the ground, and have high dental wear rates and high mesowear scores.

The data show that there was a prominent shift in average mesowear values from those re-

flexive of sharpened cusp apices (green in Fig. 1A) among the basal paraphyletic horse groups Hyracotheriinae and Anchitheriinae to those indicative of more blunted cusp apices among the dentally advanced Equinae (red, yellow, and orange in Fig. 1). The mesowear data, arranged chronologically (Fig. 1C), resemble the distribution

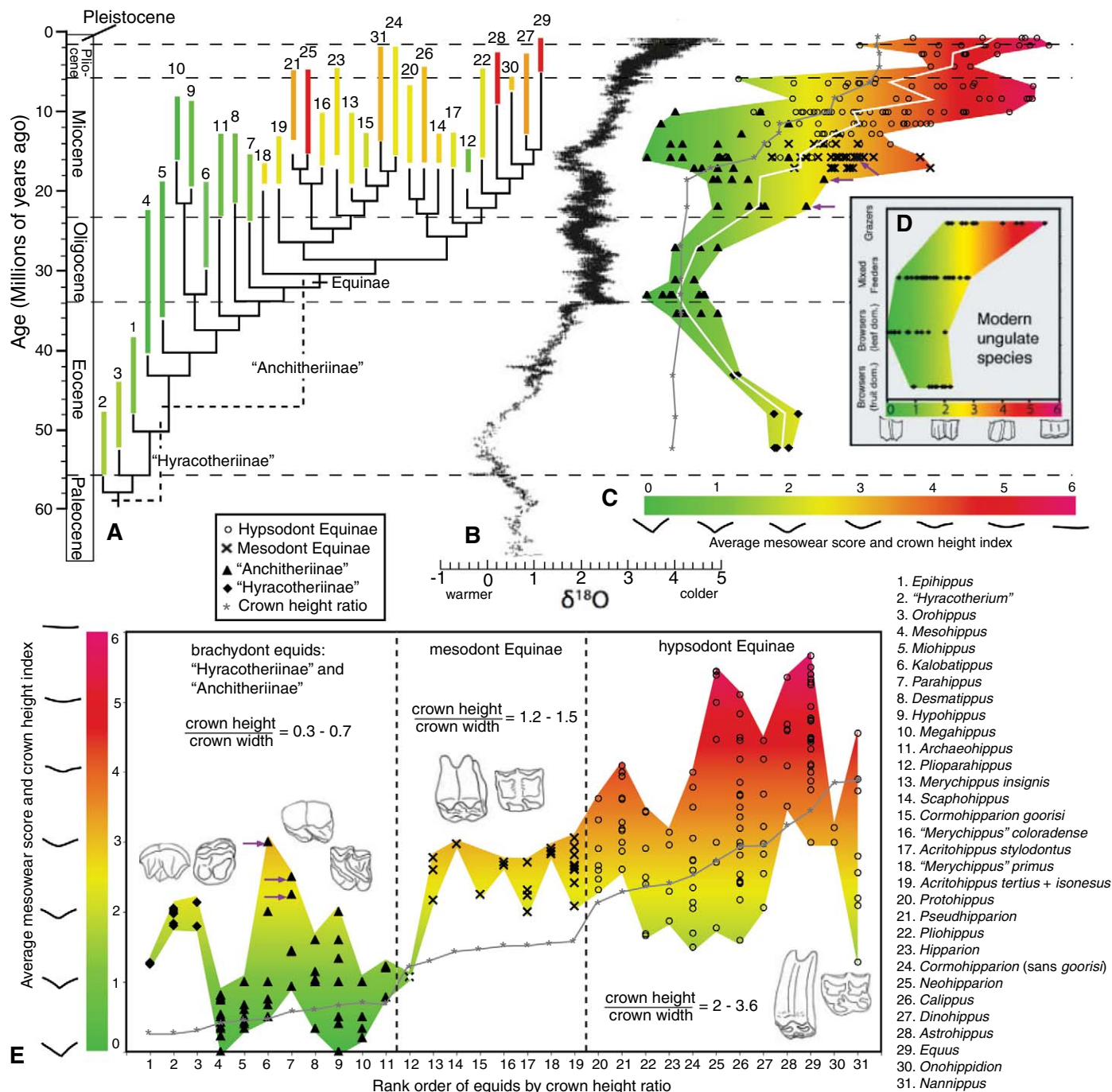


Fig. 1. Summary of mesowear for fossil Equidae (15). (A) Phylogeny of Equidae, showing stratigraphic ranges with colors representing the average mesowear score for each taxon. (B) Stacked deep-sea benthic foraminiferal oxygen isotope curve used as a proxy for global temperature (20). Increasing $\delta^{18}O$ values vary inversely with temperature. (C) Average mesowear scores (white trendline) and average crown height indices (gray trendline) for North American equids arranged temporally. Data points are positioned on the y axis (time) at the midpoint of the North American

land mammal subage. (D) Average mesowear values converted to a univariate scale for modern ungulate species arranged by diet. (E) Mesowear scores for North American equids arranged in ascending order of crown height index. Purple arrows in (C) and (E) point to samples of Anchitheriinae hypothesized to have been under the most intense selection for increased crown height. Terminal branches on the horse phylogeny (Fig. 1A) are color-coded in the same manner as the mesowear axes in Fig. 1, C and E, to reflect the average mesowear score for each taxon.

of mesowear data of modern ungulates when arranged by diet according to the hypothesized evolution of horse diets (frugivore → browser → mixed feeder → grazer) (Fig. 1D), where woody browsers have the sharpest cusps, grazers have the bluntest cusps, and mixed feeders and frugivores have more intermediate cusp morphologies. After the Eocene-Oligocene transition (EOT: 33.7 Ma), there is a directional reversal in the mesowear trend. The timing of this reversal corresponds broadly with cooling during the EOT as suggested by the $\delta^{18}\text{O}$ oxygen isotope deep-sea record (Fig. 1B) (20). The same reversal in the mesowear trend near the EOT has been reported in other North American ungulates (Merycoidodontidae) (17). The rarity of grasses during the early Eocene suggests frugivory as the best explanation for the rounded cusp-apices of the earliest horses. Frugivorous ungulates have less sharpened cusps than leafy browsers as a result of tip-crushing wear associated with feeding on hard objects and/or soil and other abrasives adhering to fallen fruit (16). Microwear and stable isotope analysis of early Eocene equids also suggest frugivorous diets for the earliest horses (21, 22). The trend toward sharper cusp apices leading up to the EOT suggests that emerging horse species tended to have diets involving less frugivory and more folivory.

Mesowear values are, on average, the lowest in the earliest Oligocene (33.7 to 32 Ma), suggesting minimally abrasive browsing diets after the prominent cooling episode of the EOT (23). Decreasing cusp sharpness in the cooler, more open habitats of the Oligocene, Miocene, Pliocene, and Pleistocene almost certainly represents continued increases in dietary abrasion, ending in the late Pleistocene with highly abrasive grazing diets like those of modern *Equus*. The alternative explanation for decreasing cusp sharpness, increasing frugivory throughout the last half of the Cenozoic, is unlikely at least for the vast majority of species; fruit would have decreased as a resource as habitats became seasonal, cooler, more arid, and more open.

Our data suggest that low-abrasion diets temporarily disappeared in the middle Miocene (23 to 19.4 Ma), a time interval during which C_3 grasslands were expanding (13), suggesting that

C_3 grasses may have become a larger part of horse diets during this time. A few available samples of brachyodont anchitheres horses begin to show mesowear scores (>2) that are equal to those of taller-crowned equine horses, including samples of *Kalobatippus*, and *Parahippus*, the genus thought to have given rise to Equinae (Fig. 1, C and E). About 17.5 to 15 Ma, horse diversity increased and was accompanied by a greater diversity of diets. The earliest Equinae show a mesowear shift that is consistent with full grazing (17.5 to 16 Ma). This is followed by the re-appearance of low-abrasion diets (16 to 14.8 Ma) for many of the dentally primitive anchitheres; the diverging mesowear patterns of anchitheres and equines during this time may have been enabled by an increase in habitat diversity around the Miocene climatic optimum (17 to 15 Ma) (24). After this interval, a high dietary diversity was sustained for millions of years, although overall levels of dietary abrasion gradually increased. Low-abrasion diets permanently disappeared among brachyodont anchitheres 10.1 to 9 Ma. C_4 grasslands expanded in the Great Plains region 6.4 to 4 Ma (25), and geochemical analysis of horse enamel indicates that at least some horses began feeding on C_4 grasses around this time (26, 27). The disappearance of intermediate-abrasion diets (5.8 to 5 Ma) occurred within this time frame, suggesting that by this time all horse populations sampled were feeding in grassy habitats. High-abrasion mesowear patterns resembling those of modern horses and zebras have persisted for the past 4 to 5 million years.

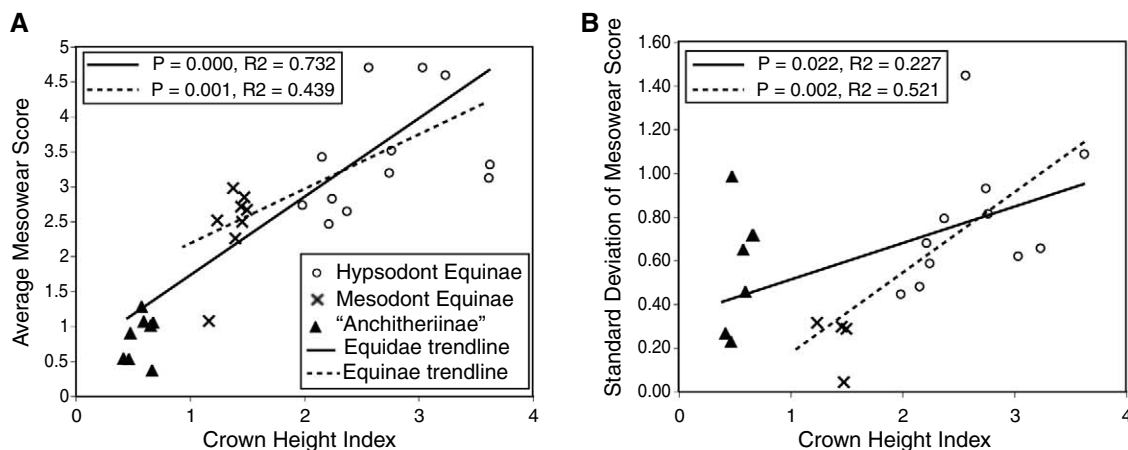
Some evolutionary changes in equid dentitions seem delayed with respect to the mesowear shifts. The most apparent of these is an early Miocene increase in dietary abrasion among anchitheres at 23 to 19.4 Ma without a notable change in crown height (Fig. 1C). The first mesodont equines appear later, about 18.8 to 17.5 Ma. Two of the mesodont equine samples are positioned well into the grazing mesowear range (red zone of Fig. 1C), well before the appearance of fully hypsodont horses (16 to 14.8 Ma). These observations are consistent with a hypothesis of adaptation in which the selective regime

precedes the morphological change. However, most equids demonstrate highly variable mesowear scores, with most samples yielding mesowear scores well below the extreme values (Fig. 1E), suggesting that selection for taller, more durable dentitions may have been episodic (13). Nonetheless, crown height and mesowear consistently show a strong statistical association when a wide variety of statistical methods are employed (Fig. 2A) (15). Although there is considerable overlap in mesowear scores between different crown height categories (Fig. 1E), hypsodont horses had higher overall mesowear scores than brachyodont and mesodont species, with many samples showing dietary abrasion greatly exceeding that of brachyodont and mesodont horses.

Crown height and variability of mesowear are also consistently significantly associated when a variety of statistical approaches are used (Fig. 2B) (15), suggesting that higher-crowned taxa were capable of adopting a wider range of diets than lower-crowned taxa. The greater variability of mesowear exhibited by hypsodont species in comparison to mesodont species supports the suggestion that hypsodonty, although possibly an adaptation to high abrasion, allows a species to become a generalist that may consume a variety of high- and low-abrasion diets (28).

There was no clear association between crown height and mesowear among anchitheres (Fig. 2), suggesting that they adopted a wider array of diets than their brachyodont dentitions indicate and had abrasion levels in some cases that were similar to those of the more dentally advanced Equinae. Small differences in crown heights among regional samples of *Anchitherium* from Europe suggest that Miocene habitat pressures were causing minor incremental increases in crown height among brachyodont horses (29). A similar pattern is not apparent in the North American anchitheres at the level considered here, where crown heights for each taxon were averaged, but we cannot rule out the possibility that analysis at a finer scale, where crown heights for individual paleopopulations are considered, might yield patterns similar to those found by (29).

Fig. 2. Plots comparing mesowear data with a dimensionless crown height index (15). **(A)** Average mesowear score versus crown height index. P and R^2 values are based on generalized least-squares regression. **(B)** Standard deviation of mesowear scores versus crown height index. Only trendlines that consistently showed significant relationships when multiple statistical methods were used are shown (15).



Mesowear patterns in horses suggest that horse diets were influenced by Cenozoic climate and its impact on vegetation and habitat structure. Horses show evidence of increased dietary abrasion as soon as grasslands became more widely available, and there is no evidence of a delay toward the shift to grazing. The temporary increase in dietary abrasion among brachyodont anchitheres beginning in the early Miocene occurred during a time during which there is evidence for increasing abundance of C₃ grasslands. However, most fossil horse samples yield highly variable mesowear scores (Fig. 1E), suggesting that most paleopopulations were experiencing rather low levels of dietary abrasion, indicating that selection for increased crown height may have been weak or absent most of the time. The famous “Great Transformation” (7) in molar crown morphology leading to the subfamily Equinae probably originated during intervals of heavy selection pressure due to pronounced increases in dietary abrasion among populations that were pioneering new habitats. A few early Miocene samples from the Great Plains (purple arrows in Fig. 1, C and E), including *Parahippus*, the genus from which Equinae originated, demonstrate mesowear values that are equal to those of later Equinae and suggest levels of dietary

abrasion that may have been extreme for their comparatively brachyodont dentitions.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/331/6021/1178/DC1
Materials and Methods

SOM Text

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Paleoindian Seafaring, Maritime Technologies, and Coastal Foraging on California’s Channel Islands

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Three archaeological sites on California’s Channel Islands show that Paleoindians relied heavily on marine resources. The Paleocoastal sites, dated between ~12,200 and 11,200 years ago, contain numerous stemmed projectile points and crescents associated with a variety of marine and aquatic faunal remains. At site CA-SRI-512 on Santa Rosa Island, Paleocoastal peoples used such tools to capture geese, cormorants, and other birds, along with marine mammals and finfish. At Cardwell Bluffs on San Miguel Island, Paleocoastal peoples collected local chert cobbles, worked them into bifaces and projectile points, and discarded thousands of marine shells. With bifacial technologies similar to those seen in Western Pluvial Lakes Tradition assemblages of western North America, the sites provide evidence for seafaring and island colonization by Paleoindians with a diversified maritime economy.

By about 13,000 years ago, Paleoindians were widespread in North America, with terrestrially focused Clovis sites found throughout the continent, especially in interior settings (1). Although a few Terminal Pleistocene sites have been found along the Pacific Coast of Peru (2, 3) and seaweeds have been dated to ~14,000 years ago at Monte Verde II in Chile (4), little is known about Paleoindian settlement, economies, and technologies along the coastlines of North America (5). Rising postglacial sea levels and coastal erosion may be responsible for this gap, but traces of early coastal set-

tlement might still be present in areas of steep bathymetry near geographic features that attracted Paleoindian peoples inland and away from now-submerged Terminal Pleistocene coastlines (6).

California’s Northern Channel Islands (Fig. 1), separated from the mainland by an oceanic strait a minimum of 7 to 20 km wide since the Last Glacial Maximum, have long been a source of claims for ancient human settlement (7). They have relatively impoverished terrestrial flora and fauna but a wealth of marine resources including seaweeds, marine mammals, shellfish, finfish, and seabirds. Human bones from the Arling-

ton Springs site on Santa Rosa Island have been dated to ~13,000 ± 200 calendar years ago (cal BP) (6–8), and a low-density shell midden at Daisy Cave on San Miguel Island dates to ~11,500 ± 200 cal BP (6), but these sites yielded no diagnostic artifacts and few faunal remains. This led some to question the fully maritime character and technological affiliation of these early Channel Island peoples (9).

Until ~10,000 years ago, the Northern Channel Islands were still connected as one large island, Santarosae. Since 13,000 cal BP, sea levels have risen by ~70 m, shrinking the size of the islands by ~65%, shifting shorelines as much as 10 km, and submerging Terminal Pleistocene coastlines where early maritime peoples most likely lived (10). By examining caves, springs, tool-stone outcrops, and other geographic features that attracted ancient maritime peoples away from Santarosae’s paleoshorelines, we have identified >50 shell middens and other sites dated

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from ~10,200 to 8000 cal BP, suggesting that a substantial human population existed on the islands in the Early Holocene (6, 11). Here, we describe three Terminal Pleistocene sites—a stratified site on Santa Rosa sealed beneath 2.5 m of alluvium and two large surficial sites on San Miguel containing five shell midden loci—that provide evidence for a diversified maritime economy and a sophisticated technology. Colonizing the Northern Channel Islands at this time required a sea voyage of 9 to 10 km (10).

CA-SRI-512W was identified on the northwest coast of Santa Rosa Island by two of the authors (J.M.E. and T.C.R.) in 2008. Situated east of the mouth of Arlington Canyon, where

the ~13,000-year-old Arlington Man skeletal remains were found in 1959 (7, 8), the site is located adjacent to the modern coast on an uplifted marine terrace ~20 m above sea level (masl). Comparison of sea level curves (12) to local bathymetric maps suggests that the site was ~5 to 7 km from the coast and ~75 masl when it was occupied. CA-SRI-512W contains a deeply buried paleosol, 30 to 40 cm thick, exposed in the eroding sea cliff (fig. S1), sealed beneath 2.5 m of alluvium containing at least five overlying soil horizons. Because the Northern Channel Islands are nearly devoid of burrowing animals, the stratigraphy of CA-SRI-512W is exceptional. Well-preserved bird bone and charcoal samples from the deeply buried paleosol—

four of them embedded in the same soil matrix as chipped stone tools, other artifacts, and the remains of marine fauna—produced AMS ¹⁴C dates ranging from ~12,000 to 11,350 cal BP (Table 1), with a most likely age range of ~11,800 to 11,500 cal BP.

At CA-SRI-512W, we collected 52 small stemmed Channel Island Barbed (CIB) points (13), 15 crescents, and numerous bird bones (table S1) from the slope below the Terminal Pleistocene paleosol. Fourteen CIB points or preforms and four crescents were found in situ in five test pits excavated in the Terminal Pleistocene paleosol. Chipped stone crescents are a relatively rare artifact found with stemmed points in some early California, Great Basin, and Columbia Plateau sites (14, 15). Other tools found in situ include biface preforms, flake tools, abundant chipped stone tool-making debris, several abraded bone tool fragments, and a large piece of red ochre with a deep groove cut across it (Fig. 2). The chipped stone tools are made primarily from local island cherts, but one piece of obsidian tool-making debris was found in situ. Geochemical analysis of this artifact indicates that it came from the West Sugarloaf flow of the Coso Volcanic Field (fig. S2) in eastern California, more than 300 km to the northeast, which suggests that Channel Island peoples participated in long-distance trade nearly 12,000 years ago.

More than 5000 bone fragments were recovered from the five test units at CA-SRI-512W, many of them burned. The faunal assemblage is dominated by birds, including several species of waterfowl and seabirds such as Canada goose (*Branta canadensis*), snow goose (*Chen*



Fig. 1. Map showing the locations of CA-SRI-512 and Cardwell Bluffs sites relative to reconstructed Terminal Pleistocene paleogeography, including –60 m (dark tan) and –50 m (light tan) submarine contours that approximate paleoshorelines at ~12,500 and 11,000 cal BP.

Table 1. AMS ¹⁴C Dates from CA-SRI-512W, CA-SMI-678, and CA-SMI-679. All dates are for single shell or charcoal fragments, measured via accelerator mass spectrometry (AMS); calendar age ranges, expressed at 1σ, were calculated in CALIB 6.0 (24), using a ΔR of 225 ± 35 for marine samples. UF, ultrafiltration. (See supporting online material for more details.)

Site (CA number): Locus and provenance	Material dated	Lab number	Measured age	Calibrated age range (cal BP)
SRI-512W: A6 paleosol	Goose bone (XAD extract)	UCIAMS-59871	10,000 ± 30	11,600–11,355
SRI-512W: A6 paleosol	Goose bone (XAD extract)	UCIAMS-59872	10,045 ± 40	11,700–11,405
SRI-512W: burned stump on slope below A6	<i>Ceanothus</i> charcoal	Beta-261353	10,090 ± 50	11,820–11,410
SRI-512W: A6 paleosol	Goose bone (UF)	OS-68030	10,150 ± 40	11,970–11,730
SRI-512W: A6 paleosol	Charred twig	UCIAMS-60751	10,155 ± 30	11,960–11,760
SRI-512W: below A6	<i>Ceanothus</i> charcoal	OS-75147	10,200 ± 45	12,010–11,820
SMI-678: Locus A, 0–5 cm	<i>Haliotis rufescens</i>	OS-59534	10,650 ± 40	11,635–11,350
SMI-678: Locus A, Unit 4, 5–10 cm	<i>Haliotis rufescens</i>	OS-75990	10,700 ± 50	11,730–11,400
SMI-678: Locus B, Unit 1, 0–5 cm	<i>Haliotis rufescens</i>	OS-80130	10,650 ± 40	11,635–11,350
SMI-678: Locus B, 0–5 cm	<i>Haliotis rufescens</i>	OS-63090	10,650 ± 55	11,655–11,340
SMI-678: Locus B, surface	<i>Haliotis rufescens</i>	OS-76006	10,750 ± 40	11,915–11,440
SMI-678: Locus C 0–5 cm	<i>Haliotis rufescens</i>	OS-59413	10,650 ± 40	11,635–11,350
SMI-678: Locus C, Unit 1, 0–10 cm	<i>Haliotis rufescens</i>	OS-63089	10,500 ± 50	11,310–11,190
SMI-678: Locus C, Unit 1, 10–20 cm	<i>Haliotis rufescens</i>	OS-76002	10,850 ± 50	12,060–11,755
SMI-678: Locus D, probe 0–5 cm	<i>Mytilus californianus</i>	OS-76005	10,850 ± 50	12,060–11,750
SMI-678: Locus D, 0–5 cm	<i>Mytilus californianus</i>	OS-76003	10,900 ± 50	12,180–11,915
SMI-678: Locus D, 10–20 cm	<i>Chlorostoma funebris</i>	OS-76004	10,900 ± 45	12,170–11,925
SMI-678: D, Unit 1, 0–5 cm	<i>Mytilus californianus</i>	OS-80129	10,950 ± 45	12,240–12,000
SMI-679SE: Tegula feature	<i>Chlorostoma funebris</i>	OS-63091	10,750 ± 55	11,990–11,430
SMI-679SE: Tegula feature	<i>Mytilus californianus</i>	OS-76008	10,800 ± 45	11,955–11,710
SMI-679SE: Unit 1, 0–5 cm	<i>Haliotis rufescens</i>	OS-80131	10,800 ± 45	11,955–11,710
SMI-679SE: Unit 1, 15 cm	<i>Haliotis rufescens</i>	OS-80171	10,800 ± 40	11,950–11,720
SMI-679SE: Tegula feature	Charred twig	OS-70638	10,900 ± 45	12,920–12,630

caerulescens), cormorant (*Phalacrocorax* spp.), and albatross (*Phoebastria albatrus*). The CIB points and chipped stone crescents seem likely

to have been used to hunt birds, but pinniped and undifferentiated marine mammal bones were also recovered, along with smaller numbers of

bones from rockfish (*Sebastes* spp.), greenling (*Hexagrammidae*), sculpin (*Cottidae*), surfperch (*Embiotocidae*), and the herring/sardine family



Fig. 2. Paleocoastal artifacts from CA-SRI-512W. Channel Island Barbed (CIB) points at left and three crescents in center column are from slope below eroding A6 paleosol; sawn red ochre (lower center), abraded bone tool fragments (upper right), projectile points, and crescents at right were found in situ in test units. Scale bar, 1 cm per square. [Photo by J. Erlandson]

Fig. 3. Chert projectile points from CA-SMI-678 and CA-SMI-679. Center column, five eccentric crescents (top to bottom: SMI-679-39, -214, -67, -5, and -341). Left columns, Amol points (top to bottom, column 1: 678-58, 679-24, 679-256; column 2: 678-722, 679-28, 678-38). Right columns, CIB points (column 4, top to bottom: 679-255, 679-216, 679-300; column 5: 679-215, 678-101, 678-86). SMI-678-722 was found in situ within shell midden stratum dated to ~12,240 to 11,750 cal BP. Scale bar, 1 cm per square. [Photo by J. Erlandson]



(Clupeidae) (table S3). On the slope below the site, we found a burned tibiotarsus fragment from an extinct flightless goose, *Chendytes lawi* (16). No marine shells have been recovered from CA-SRI-512W.

The relatively small size of this Paleocoastal site (which is exposed for about 60 m along the modern sea cliff), along with the density and diversity of artifacts and faunal remains, suggests that it served as a seasonal hunting camp, probably occupied by fairly mobile hunter-gatherers. The abundance of geese implies that the site was occupied in winter (17), but it may also have been used in other seasons. Situated just east of Arlington Canyon, the largest drainage on Santa Rosa Island, the site may have been located downwind (south-east) of a broad, marshy canyon bottom. Wetlands in this canyon would have been an ideal location to hunt waterfowl and seabirds attracted to the island by the lack of large terrestrial predators.

Near Cardwell Point on San Miguel Island, naturally occurring chert cobbles can be found scattered across a raised marine terrace at 65 to 75 masl. Here we identified two large and heavily eroded lithic scatters (CA-SMI-678 and -679) that were situated ~1 to 2 km from the coast and ~125 masl during the Terminal Pleistocene. At the Cardwell Bluffs sites, in an area ~500 m long and 300 m wide, we collected more than 400 whole or broken bifaces (table S2) and identified remnants of five discrete shell middens where faunal remains and artifacts were found in situ. AMS ^{14}C dates from well-preserved marine shells from four middens at CA-SMI-678 ranged from ~12,250 to 11,200 cal BP. Four dates on marine shells from a small midden at CA-SMI-679 average ~11,850 cal BP. A charred twig from this midden, dated to ~13,000 cal BP, may indicate an earlier human occupation contemporary with Arlington Springs. Because of potential problems with natural wildfires and the clearer cultural origin of the marine shells, however, we believe that the shell dates better estimate the age of the CA-SMI-679 midden.

Stratigraphic profiles at CA-SMI-678 and CA-SMI-679 demonstrate that portions of the sites were once buried under a thin sheet of dune sand, probably until the 19th to 20th centuries when overgrazing by introduced livestock caused heavy erosion. On eroded surfaces, Paleocoastal artifacts were found in several clusters interspersed with intact site remnants, suggesting that multiple occupations of the sites occurred over a millennium or more. Twelve test pits excavated in intact site remnants produced thousands of chipped stone artifacts, nearly 10 kg of marine shell (tables S4 and S5), but no animal bone.

The chipped stone bifaces from Cardwell Bluffs ranged from preforms to finished or broken projectile points (Fig. 3). Preforms consisted primarily of bifaces broken during manufacture of points or knives, whereas the finished artifacts were mostly basal point fragments that appear to have been discarded during repair of hunting gear. We collected 31 crescents, including lunate forms and “eccentric” varieties with notches and projections

on one side. Stemmed points were more abundant, including 32 CIB points of various sizes and shapes, some with elongated stems and pronounced barbs, others with shorter stems or barbs and serrated blade margins. We also recovered 23 Amol points, a previously unknown stemmed point type that lacks barbs although most examples have serrated blade margins. Variation in the two stemmed point types may result from the reworking and recycling of broken points. Most of the points, preforms, and bifaces come from eroded site surfaces, but many were found around the margins of the dated shell middens, and three bifaces and an Amol point were found in situ in the ~12,100-year-old shell midden at CA-SMI-678D.

The marine shell from CA-SMI-678 demonstrates that Paleoindians harvested a variety of shellfish from rocky intertidal and kelp forest habitats, including red abalone (*Haliotis rufescens*), giant chiton (*Cryptochiton stelleri*), mussel (*Mytilus californianus*), black turban snail (*Chlorostoma [Tegula] funebris*), and crabs (table S4). The abundance of red abalone and giant chiton, and the near absence of black abalone (*H. cracherodii*), suggests that sea surface temperatures were cooler than today. An 11,850-year-old basin-shaped midden feature at CA-SMI-679, ~1 m in diameter and 5 to 15 cm thick, was dominated by black turban shells, a small intertidal gastropod easily gathered but laborious to process. Pitted stones found nearby were probably used as anvils to crack turban shells and extract their meat. A more extensive shell midden about 3 to 4 m to the west contained a more balanced shellfish assemblage (table S5) similar to that of CA-SMI-678.

Along with Daisy Cave and Arlington Springs, these Paleocoastal sites are preserved because they were situated on upland landscapes distant from submerged Terminal Pleistocene shorelines—interior localities where Paleocoastal people were attracted by sources of tool stone, fresh water, shelter, and possibly a marsh near CA-SRI-512. The artifact assemblages from CA-SRI-512W and Cardwell Bluffs provide a window into the technologies used by maritime Paleoindians on California's Channel Islands. Early projectile point technologies along the southern California Coast were long thought to have been relatively crude (18, 19), but the finished points and crescents from these island sites are finely made—thin and highly symmetrical, with delicate barbs, serrations, or notches. Although the faunal assemblages from Cardwell Bluffs and CA-SRI-512W are very different, together they demonstrate that Paleocoastal peoples on the Northern Channel Islands took advantage of a diverse array of marine and aquatic resources, including shellfish, waterfowl and seabirds, sea mammals, and finfish.

If Arlington Springs is included, the earliest Paleocoastal Channel Island sites are contemporary with Clovis and Folsom sites of the continental interior (6, 8, 20). The island sites provide evidence for Terminal Pleistocene seafaring, island colonization, and a diversified maritime economy, adding to the variability of Paleoindian adapta-

tions in the Americas. The stemmed points and crescents dated as early as 12,200 cal BP link these early island assemblages to those found in interior Western Pluvial Lakes Tradition (WPLT) sites found around many lakes and marshes in North America's Far West (15). Stemmed point fragments have also been recovered in the basal levels of Paisley Caves, dated to ~14,300 cal BP (21), and the Paleocoastal stemmed points and crescents from the Channel Islands seem unlikely to be descended from Clovis. Such WPLT assemblages may provide a logical technological link among Terminal Pleistocene stemmed point traditions of Northeast Asia (22), the Pacific Northwest, and possibly early stemmed point traditions widely distributed in South America (23).

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Pseudomonas sax Genes Overcome Aliphatic Isothiocyanate-Mediated Non-Host Resistance in *Arabidopsis*

Jun Fan,^{1*†} Casey Crooks,^{1,2†} Gary Creissen,¹ Lionel Hill,¹ Shirley Fairhurst,¹ Peter Doerner,^{3,4*} Chris Lamb^{1‡}

Most plant-microbe interactions do not result in disease; natural products restrict non-host pathogens. We found that sulforaphane (4-methylsulfinylbutyl isothiocyanate), a natural product derived from aliphatic glucosinolates, inhibits growth in *Arabidopsis* of non-host *Pseudomonas* bacteria in planta. Multiple *sax* genes (*saxCAB/F/D/G*) were identified in *Pseudomonas* species virulent on *Arabidopsis*. These *sax* genes are required to overwhelm isothiocyanate-based defenses and facilitate a disease outcome, especially in the young leaves critical for plant survival. Introduction of *saxCAB* genes into non-host strains enabled them to overcome these *Arabidopsis* defenses. Our study shows that aliphatic isothiocyanates, previously shown to limit damage by herbivores, are also crucial, robust, and developmentally regulated defenses that underpin non-host resistance in the *Arabidopsis*-*Pseudomonas* pathosystem.

Non-host resistance is the ability of most plant species to resist microbes or viruses that are successful pathogens on other plants. It is the most prevalent form of plant disease resistance, is durable and effective against a broad range of potential pathogens, but our understanding of its molecular basis is still poor (1–3). Plants generate a huge diversity of natural products, with multiple roles in defense, communication, and development (4). Preformed natural products provide chemical barriers to phytopathogenic fungi (5–7) and are deterrents in plant-herbivore interactions (8). However, their role in restricting bacterial host range remains obscure, as do the bacterial mechanisms involved in breaching natural product-mediated host defenses. To better understand fundamental host-pathogen biology and to inform the development of sustainable field resistance to major crop diseases (9), we sought to define plant components conferring non-host resistance as well as strategies used by virulent pathogens to overcome resistance barriers.

We observed that extracts from naïve *Arabidopsis* plants inhibited the growth of most pathogens of *Pseudomonas syringae* for which *Arabidopsis* is not a host. In contrast, *P. syringae* pathovars *maculicola* (*Psm*) ES4326 and *tomato*

(*Pst*) DC3000, which are pathogenic on *Arabidopsis*, grew well on rich media supplemented with host extract from Col-0 (Table 1) or other accessions (table S1). Using *Arabidopsis*-*P. syringae* as a model system to dissect plant resistance to non-host pathogens, we screened a *Psm* ES4326 genomic library for genes conferring resistance in *Escherichia coli* to *Arabidopsis* extracts (Table 1) (10). A single operon, designated *sax* (survival in *Arabidopsis* extracts), allowed *E. coli* to grow on *Arabidopsis* extracts. In *E. coli*, *saxA* and *saxC* are together necessary for resistance: The absence of either resulted in susceptibility, whereas the absence of *saxB* reduced bacterial growth on extracts (fig. S1A). *SaxA* has a predicted secretory signal peptide (11) and, although related to class B β -lactamases (12, 13), it is unable to confer resistance to eight representative β -lactam antibiotics (fig. S2A), indicating that *SaxA* activity is distinct. *SaxB* is related to isochorismatase. *SaxC* is a highly conserved member of the AraC/XylS family of transcriptional regulators found in diverse prokaryotes and involved in carbon metabolism, stress response, and pathogenesis (14). Analysis of 35 plant-associated *P. syringae* genomes showed that only *Arabidopsis* pathogens have *saxA*-like genes with $\geq 90\%$ nucleic acid sequence identity to *Psm* ES4326 *saxA* (table S2 and fig. S3).

Non-host resistance is durable, and hence it is unlikely to be overwhelmed by a single mechanism. Indeed, deletion of *saxAB* genes in *Pst* DC3000 or *Psm* ES4326 had little impact on bacterial growth in *Arabidopsis* extracts (Table 1). To identify additional protective mechanism(s), we screened for compromised bacterial growth in *Arabidopsis* extracts after transposon mutagenesis of *Pst* Δ *saxAB* (10). Two putative multidrug re-

sistance (MDR) efflux genes were identified, with a third similar system revealed in *Pst* DC3000 by genome analysis (10); these were designated *saxF*, *saxD*, and *saxG*, respectively (fig. S1B). They form a subgroup among the nine resistance-nodulation-division (RND) efflux systems predicted in the *Pst* DC3000 genome (15), which function to extrude a wide range of substrates including antibiotics and host-derived molecules (16). We sequentially deleted *saxF/D/G* from the *Pst* Δ *saxAB* background, which resulted in progressively increased sensitivity (Table 1); hence, these genes were required for robust resistance to *Arabidopsis* extracts. Deletion of *saxAB*, *saxF/D/G*, or *saxAB/F/D/G* did not impair growth in rich medium (Fig. 1A), indicating that they are not essential. Thus, *sax* genes have distinct but complementary roles in *Pseudomonas* resistance to *Arabidopsis* extracts.

We purified the *Arabidopsis* antimicrobial compound restricting non-host *Pseudomonas*

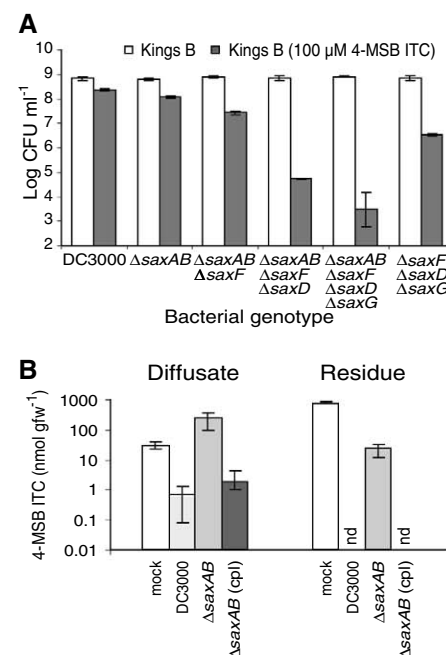


Fig. 1. *sax* genes in *Pst* DC3000 protect against *Arabidopsis*-derived isothiocyanates. (A) *sax* genes are synergistically required for bacterial resistance to isothiocyanates. Bacterial strains were inoculated into Kings B medium with or without sulforaphane and grown overnight. (B) Bacterial infection released sulforaphane (4-MSB ITC) into apoplastic diffusates. *Arabidopsis* leaf discs were vacuum-infiltrated with bacteria [optical density at 600 nm (OD₆₀₀) = 0.1] and incubated at 23°C for 48 hours before analysis. Data are means $\pm$ SD; cpl, episomal complementation with the *sax* operon; gfw, gram fresh weight; nd, not detected.

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from Col-0 extracts (10) and identified it as sulforaphane [4-methylsulfinylbutyl isothiocyanate (4-MSB ITC)] (fig. S5). Aliphatic isothiocyanates are breakdown products derived from glucosinolates, a class of sulfur- and nitrogen-containing natural products widely distributed in crucifers; *Arabidopsis* plants produce a variety of glucosinolates, with extensive variation in leaf and root content across different accessions (17). In Col-0, 4-MSB glucosinolate predominantly accumulates in rosette leaves (18); however, *Arabidopsis* plants with loss-of-function alleles of MYB28 and MYB29 transcription factors, but not Col-0 plants transformed with 35S::*saxA*, lack aliphatic glucosinolates (19, 20) (table S3). A panel of virulent and non-host bacteria showed a higher median inhibitory concentration (IC₅₀) for sulforaphane in the former (table S5).

Antimicrobial activity of sulforaphane in vitro has been reported (21), but its role in disease

resistance in planta is unknown. We tested the protective effect of the *sax* operon against a panel of isothiocyanates derived from glucosinolates prevalent in *Arabidopsis* and other crucifers. *E. coli* carrying the *sax* operon was more tolerant than the vector control to all five tested compounds (fig. S2B), indicating that *saxCAB* genes are active against a broad spectrum of isothiocyanates. Deletion of *saxAB*, *saxF/D/G*, or *saxAB/F/D/G* progressively impaired growth in rich medium supplemented with sulforaphane, or in extracts from *myb28/29* plants supplemented with sulforaphane, but not in extracts from Col-0 plants transgenic for 35S::*saxA* (Fig. 1A and Table 1); these findings indicate that SaxA is sufficient for detoxification of sulforaphane and other aliphatic isothiocyanates (Fig. 2A and fig. S2B). Treatments with *Arabidopsis* extracts or with sulforaphane induced *saxA* and *saxF* expression (fig. S4). These findings indicate a broader

role for aliphatic isothiocyanates in plant-biotic interactions than previously understood.

Glucosinolate breakdown is activated by tissue damage after herbivory; the subsequent accumulation of aliphatic isothiocyanates is a major determinant of plant-herbivore interactions (20, 22, 23). However, bacterial infections do not normally cause similar tissue damage. We examined whether infection released isothiocyanates into the apoplastic space that hemibiotrophic bacteria colonize (10). Relative to mock treatments, sulforaphane levels were reduced in diffusate and residue samples treated with wild-type *Pst* DC3000, whereas infection with the *Pst*Δ*saxAB* strain increased sulforaphane levels in diffusate (Fig. 1B). Thus, *Pst* DC3000 infection triggers glucosinolate degradation and release of isothiocyanates into the apoplast, and this *Arabidopsis* response is countered by *Pst* DC3000 in a *sax*-dependent manner.

Table 1. Inhibitory activity of *Arabidopsis* extracts on bacterial growth in vitro. Bacterial growth was determined 24 hours after inoculation, or as indicated otherwise. Data are means ± SD. *Pst* strains are from this study; other strain sources are as indicated. Strains E54326, M1, M2, M3, M5, M6, and DC3000 grow on crucifers. "With ITC" denotes *myb* mutant plant extract supplemented with authentic sulforaphane (4-MSB ITC) at a concentration of 200 μg ml⁻¹. Statistical significance of growth inhibition (rightmost column): **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (two-tailed *t* test; other values are not significant).

Bacterial strain and source	log(CFU ml ⁻¹)				
	Col-0	35S:: <i>saxA</i>	<i>myb28-1/myb29-1</i>		
			Without ITC	With ITC	Inhibition
<i>P. syringae</i> pathovars					
pv. <i>maculicola</i> ES4326*	8.9 ± 0.05	9.1 ± 0.10	9.2 ± 0.03	7.6 ± 0.08	1.6**
pv. <i>maculicola</i> M1†	9.3 ± 0.10	9.2 ± 0.05	9.3 ± 0.10	9.1 ± 0.18	0.2
pv. <i>maculicola</i> M2†	8.5 ± 0.06	8.9 ± 0.16	8.8 ± 0.07	8.6 ± 0.12	0.2
pv. <i>maculicola</i> M3†	8.8 ± 0.09	9.2 ± 0.13	9.0 ± 0.01	7.8 ± 0.09	1.2**
pv. <i>maculicola</i> M5†	8.1 ± 0.07	7.4 ± 0.02	7.0 ± 0.03	7.0 ± 0.10	0
pv. <i>maculicola</i> M6†	9.2 ± 0.11	9.4 ± 0.19	9.2 ± 0.27	9.3 ± 0.03	−0.1
pv. <i>tomato</i> DC3000*	8.9 ± 0.03	9.0 ± 0.07	8.6 ± 0.10	7.9 ± 0.05	0.7*
pv. <i>tomato</i> T1‡	4.3 ± 0.07	8.9 ± 0.06	8.7 ± 0.05	3.5 ± 0.33	5.2**
pv. <i>apii</i> ATCC9654*	7.2 ± 0.08	9.3 ± 0.24	9.1 ± 0.17	4.5 ± 0.05	4.6***
pv. <i>coronafaciens</i> KN221‡	8.5 ± 0.13	9.5 ± 0.10	9.4 ± 0.00	7.2 ± 0.34	2.2*
pv. <i>glycinea</i> race 4*	5.5 ± 0.20	7.7 ± 0.00	7.5 ± 0.11	4.3 ± 0.03	3.2***
pv. <i>glycinea</i> race 5*	6.4 ± 0.16	7.5 ± 0.12	7.4 ± 0.16	5.4 ± 0.07	2.0**
pv. <i>lachrymans</i> ATCC 7386*	6.9 ± 0.01	8.6 ± 0.07	8.3 ± 0.03	<3.3§	5.0***
pv. <i>phaseolicola</i> 1448A‡	8.8 ± 0.08	9.5 ± 0.03	9.3 ± 0.12	7.7 ± 0.09	1.6**
pv. <i>syringae</i> B728A†	7.7 ± 0.07	9.5 ± 0.03	9.5 ± 0.02	6.3 ± 0.17	3.2**
pv. <i>syringae</i> PSC1B‡	3.3 ± 0.10	7.3 ± 0.11	7.0 ± 0.10	4.5 ± 0.08	2.5**
pv. <i>tabaci</i> 6605†	5.4 ± 0.53	8.8 ± 0.03	8.5 ± 0.30	5.7 ± 0.11	2.8**
<i>Xanthomonas</i> spp.					
<i>X. perforans</i> race 4†	8.0 ± 0.30	9.2 ± 0.09	8.9 ± 0.01	<3.3§	5.6***
<i>X. campestris</i> pv. <i>vesicatoria</i> 69-1†	6.0 ± 0.06	8.7 ± 0.13	8.4 ± 0.03	<3.3§	5.1***
<i>E. coli</i>					
DH5α*	4.6 ± 0.08	8.9 ± 0.21	9.2 ± 0.14	4.7 ± 0.10	4.5***
<i>Pst</i> -derived mutants and transformants					
Δ <i>saxAB</i>	8.7 ± 0.07	9.0 ± 0.04	8.8 ± 0.05	7.5 ± 0.15	1.3**
Δ <i>saxF/D/G</i>	<3.3§	9.0 ± 0.10	8.8 ± 0.04	<3.3§	5.5***
Δ <i>saxF/D/G</i> (<i>saxF</i>)	8.8 ± 0.15	8.9 ± 0.08	8.7 ± 0.12	8.1±0.2	0.6*
Δ <i>saxAB/F/D/G</i>	<3.3§	9.0 ± 0.12	9.0 ± 0.00	<3.3§	5.4***
Δ <i>saxAB/F/D/G</i> (<i>saxF</i> + <i>saxCAB</i>)	8.9 ± 0.11	8.8 ± 0.16	8.8 ± 0.31	8.9 ± 0.03	−0.1
T1	6.0 ± 0.07	8.8 ± 0.12	8.2 ± 0.27	3.5 ± 0.21	4.7**
T1 (<i>saxCAB</i>)	8.7 ± 0.22	8.8 ± 0.11	8.0 ± 0.21	8.7 ± 0.12	−0.7

*Strain from Lamb lab stock. †Strain from C. Zipfel. ‡Strain from K. Sohn. §No colony was detected from 1/10 dilution of the culture. ||Bacterial growth of these strains was determined at 48 hours, except for Δ*saxAB*.

To determine whether the isothiocyanate resistance of *Pst* DC3000 plays an important role in pathogenesis on *Arabidopsis*, we monitored bacterial growth after infiltration of *Pst* DC3000 or *saxAB/F/D/G*. Consistent with a previous report (24), we found that sulforaphane levels in apoplastic diffusates of young leaf discs were higher by a factor of ~100 than in old leaf discs infected with the *Pst*Δ*saxAB* strain (Fig. 3A), which suggests differences in their glucosinolate-mediated

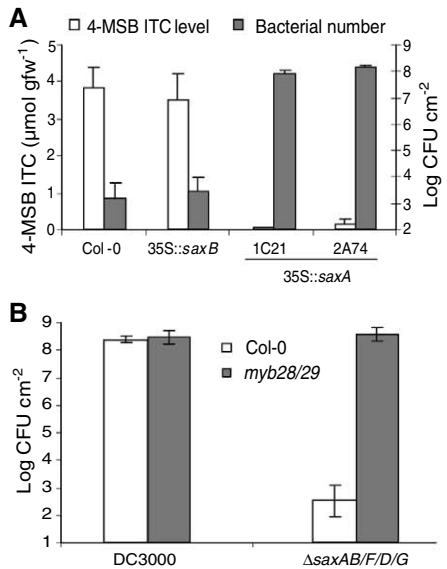


Fig. 2. Accumulation of aliphatic glucosinolates and isothiocyanates is necessary for suppressing the quintuple *sax* mutant in young *Arabidopsis* leaf tissue. (A) Representative *Arabidopsis* transgenic plants expressing the bacterial *saxA* gene fail to produce high levels of sulforaphane (4-MSB ITC) in crude extracts and are fully susceptible to the quintuple *sax* mutant. (B) Disruption of aliphatic glucosinolate biosynthesis abolished resistance to the quintuple *sax* mutant in young *Arabidopsis* leaf tissue. In (A) and (B), discs from young rosette leaves were treated as described (Fig. 1B) and bacterial titer analyzed 3 days after inoculation. Data are means ± SD.

Fig. 3. *sax* genes are required for *Pst* DC3000 survival during infection of young *Arabidopsis* leaf tissue releasing high levels of sulforaphane (4-MSB ITC). (A) Levels of 4-MSB ITC differed significantly between young and old *Arabidopsis* leaves. (B) The quintuple *sax* mutant is unable to maintain infection on young *Arabidopsis* leaf tissue. The conditions for these bacterial infection experiments were the same as described in Fig. 1B. Data are means $\pm$ SD; dpi, days post-inoculation.

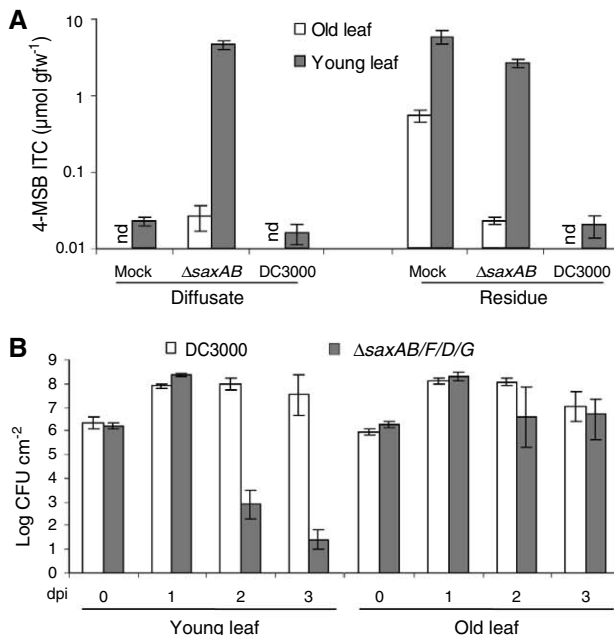
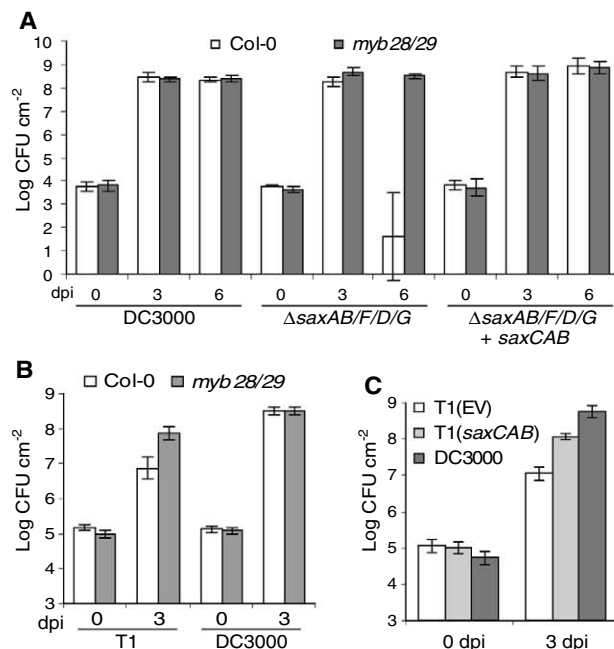


Fig. 4. Aliphatic isothiocyanates restrict bacterial growth in planta. (A) Young leaves of Col-0 and *myb28/29* plants were syringe-infiltrated with bacteria ($\text{OD}_{600} = 0.001$). (B) Aliphatic glucosinolate biosynthesis is required for growth suppression of the non-host *Pst* T1 strain. (C) The *saxCAB* operon promoted growth of *Pst* T1 on Col-0 plants. In (B) and (C), young leaves of 5- to 6-week-old plants were infiltrated with bacteria ($\text{OD}_{600} = 0.02$). Data are means $\pm$ SD; EV, empty vector control.



defense capacity. Relative to wild-type *Pst* DC3000, bacterial titer was reduced by six orders of magnitude in the $\Delta\text{saxAB/F/D/G}$ strain in the young but not the old leaf discs (Fig. 3B). In planta assays with much lower inoculum density (Fig. 4A) or when inoculated by spraying (fig. S7) produced similarly reduced levels of growth of the $\Delta\text{saxAB/F/D/G}$ strain. Thus, *sax* genes are important for *Pst* DC3000 to penetrate host barriers and maximize its population in the young leaves.

We then examined transgenic *Arabidopsis* plants overexpressing *sax* genes to examine whether *sax*-dependent reactions are sufficient to negate bacterial restriction by the host. The levels of major glucosinolates were unchanged in the *saxA*-expressing transgenic plants (table S3), suggesting

that SaxA targets steps after glucosinolate breakdown. Sulforaphane levels in extracts of young leaves from *saxA* transgenic plants were lower than those from wild-type Col-0 and *saxB* transgenic plants by a factor of >20 (Fig. 2A). Growth inhibition of the $\Delta\text{saxAB/F/D/G}$ mutant observed in leaves from Col-0 plants was lost in *saxA* transgenic plants (Fig. 2A) and in the *myb28/29* background (Fig. 2B, Fig. 4A, and fig. S7). These observations indicate that aliphatic isothiocyanates are potent host defense effectors that restrict bacterial populations. However, we also observed that the titer of the quintuple *sax* mutant did not fall until necrotic symptoms had developed, implying that the isothiocyanate-mediated defense might be potentiated by cell death in the host.

To examine the broader role of aliphatic isothiocyanates in restricting non-host pathogens, we tested an expanded panel of crucifer and non-crucifer pathogens (Table 1) with *Arabidopsis* extracts. In extracts prepared from wild-type Col-0 plants, all the crucifer pathogens tested were able to grow well, whereas the titer of the noncrucifer strains varied between $10^{3.3}$ and $10^{8.8}$ CFU/ml and showed a clear trend of growth inhibition. However, when grown in extracts from *myb28/29* or *35S::saxA* plants, growth inhibition was attenuated in the noncrucifer pathogens. After supplementing extracts of *myb28/29* plants with sulforaphane (Table 1) or iberin (3-methylsulfinylpropyl isothiocyanate) (table S4), restriction of noncrucifer pathogens was restored, whereas we observed only small growth differences of the crucifer pathogens between different treatments. Hence, aliphatic isothiocyanates are essential for *Arabidopsis* extracts to restrict the growth of many noncrucifer bacterial pathogens.

We examined whether *saxCAB* was sufficient to allow non-host pathogens to overcome *Arabidopsis* aliphatic isothiocyanate-dependent defenses. A tomato isolate of *P. syringae*, *Pst* T1, which is not pathogenic in *Arabidopsis* (25, 26), does not grow on *Arabidopsis* extracts (Table 1). *saxA* is absent from a homologous region of the *Pst* T1 genome (fig. S6A), whereas its *sax*-related efflux systems are $\geq 99\%$ identical to those in *Pst* DC3000 (fig. S6B). When challenged with *Pst* T1, young leaves of *myb28/29* plants supported ~ 10 times as much bacterial growth as did Col-0 (Fig. 4B). Transformation of *Pst* T1 with *Psm* ES4326 *saxCAB* rescued *Pst* T1 growth in *Arabidopsis* extracts containing sulforaphane (fig. S8) and enhanced growth in young Col-0 leaves by a factor of ~ 10 (Fig. 4C). Similar results were obtained with the non-host pathovar *Ps* *apii* (ATCC 9654) (Table 1 and fig. S9). We conclude that isothiocyanate-mediated defense effectively limits infection by non-host pathogenic *Pseudomonas* bacteria and that *sax* genes strongly enhance *Pseudomonas* virulence in *Arabidopsis*.

Indolic glucosinolates are required for *Arabidopsis* to prevent penetration of non-adapted powdery mildew strains (27) and to activate callose deposition as an innate immune response (28). Our findings show that *Pseudomonas* pathogens require *sax* gene-dependent mechanisms to overwhelm aliphatic isothiocyanate-mediated non-host resistance in *Arabidopsis*. This and other work (27, 28) shows how non-host resistance, unlike many examples of major *R* gene resistance to specific pathogen genotypes, can be resilient, durable, and broadly effective against many potential pathogens, thereby revealing emergent principles for engineering sustainable field resistance for major crop diseases.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/331/6021/1185/DC1

Materials and Methods

Figs. S1 to S9

Tables S1 to S6

References

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A Sorting Platform Determines the Order of Protein Secretion in Bacterial Type III Systems

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Bacterial type III protein secretion systems deliver effector proteins into eukaryotic cells in order to modulate cellular processes. Central to the function of these protein-delivery machines is their ability to recognize and secrete substrates in a defined order. Here, we describe a mechanism by which a type III secretion system from the bacterial enteropathogen *Salmonella enterica* serovar Typhimurium can sort its substrates before secretion. This mechanism involves a cytoplasmic sorting platform that is sequentially loaded with the appropriate secreted proteins. The sequential loading of this platform, facilitated by customized chaperones, ensures the hierarchy in type III protein secretion. Given the presence of these machines in many important pathogens, these findings can serve as the bases for the development of novel antimicrobial strategies.

Bacterial pathogens have evolved the capacity to deliver effector proteins into target host eukaryotic cells (1). Various protein-delivery machines have been described, including the so-called type III, type IV, and type VI secretion systems (2–5). Type III protein secretion systems (T3SSs) are among the most complex protein-secretion systems known (2, 5). The needle complex, a central component of these systems, serves as a conduit for the secreted proteins as they pass through the bacterial envelope (6). Other essential elements of T3SSs are several cytoplasmic proteins of unknown function and the so-called export apparatus. A group of proteins known as “translocases” facilitate the delivery of the effector proteins through the eukaryotic host cell membrane (7, 8). Protein secretion must be precisely coordinated to ensure the deployment of components of the needle complex, followed by the deployment of the pro-

tein translocases and the delivery of effector proteins (9–16). Interfering with the hierarchy of secretion results in loss of T3SS function (17).

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) encodes a T3SS, which mediates its interactions with the intestinal epithelium (18). We investigated the function of cytoplasmic components of this T3SS, which are essential for its function. One of these proteins is SpaO (19), a conserved component of T3SSs that shares limited amino acid sequence similarity to components of a flagellar substructure known as the C ring (20, 21). We carried out a bacterial subcellular fractionation to localize SpaO (22) and found a substantial proportion (~20%) in high-speed centrifugation pellets of total cell lysates (Fig. 1A), with a sizable amount of SpaO present in the same sucrose gradient fractions as the needle complex (Fig. 1B). However, a substantial proportion of SpaO was also detected in fractions that did not contain the needle complex (Fig. 1B). Furthermore, the subcellular localization of SpaO did not appreciably change in a mutant strain lacking the needle complex and membrane protein components of the export apparatus (Fig. 1C),

and considerable amounts of SpaO remained pelletable even after treatment with a detergent concentration that should solubilize membranes (Fig. 1D). Thus, SpaO appears to form a high molecular weight complex even in the absence of all the bacterial envelope components of the T3SSs. To further characterize the SpaO complex, we used blue native polyacrylamide gel electrophoresis (BN-PAGE) (23). SpaO formed a large, heterogeneous complex whose size did not change much in the absence of the needle complex or the membrane protein components of the export apparatus (Fig. 1E).

To identify components of the SpaO complex, we immunoprecipitated SpaO from whole-cell lysates or sucrose gradient fractions, which should only contain SpaO associated with the high molecular weight complex. Immunoprecipitated proteins were identified by liquid chromatography–mass spectrometry (LC-MS/MS) after one-dimensional SDS-PAGE (Fig. 2A and fig. S1) or two-dimensional BN-PAGE (Fig. 2B and fig. S2). We detected several T3SS-related proteins, including components of the needle complex and export apparatus; the cytoplasmic proteins OrgA, OrgB, and InvC; and the protein translocases SipB, SipC, and SipD. OrgA and OrgB were also found to be part of a high molecular weight complex similar in size to the SpaO complex (Fig. 2, C and D). A preparation from cells lacking OrgA and OrgB showed a reduced amount of SpaO in the high molecular weight complex, suggesting that these proteins may be required for the efficient assembly and/or stability of the SpaO complex (fig. S3). Absence of the adenosine triphosphatase (ATPase) InvC, by contrast, had no effect on the abundance of SpaO in the high molecular weight complex (fig. S3). Thus, the cytoplasmic proteins SpaO, OrgA, and OrgB form part of a high molecular weight multiprotein complex that can include components of the needle complex and export apparatus.

The bacterial translocases SipB, SipC, and SipD were also readily detected in SpaO immunoprecipitates (Fig. 2, A and B, and figs. S1 and S2),

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Fig. 1. SpaO forms a high molecular weight complex. (A and B) Subcellular fractionation of SpaO. (A) A whole-cell lysate (WCL) of a *S. Typhimurium* strain expressing FLAG-epitope-tagged SpaO was separated into soluble and pelletable fractions by high-speed centrifugation, and the presence of SpaO in the different fractions was probed by immunoblotting with an antibody against FLAG. (B) The pellet fraction was then separated by sucrose gradient centrifugation, and the different gradient fractions were probed for the presence of SpaO (green) and needle complex components (red) by immunoblotting and imaging with the Odyssey system (Li-Cor Bioscience). (C) Comparison of the distribution of SpaO in wild-type and the $\Delta invA \Delta spaPQRS \Delta prgHIJK$ isogenic mutant derivative, which lacks the needle complex and membrane protein components of the export apparatus. The distribution of SpaO in the different fractions of a sucrose gradient was probed by immunoblot as in (A). (D) Localization of SpaO after different treatments. Pellet fractions of SpaO obtained from the indicated strains were subjected to different treatments (as indicated), and localization of SpaO after high-speed centrifugation was determined by immunoblot analysis of the pellet and soluble fractions. (E) Analysis of the SpaO complex by BN-PAGE. Pellet fractions from a wild-type *S. Typhimurium* encoding a FLAG-epitope-tagged SpaO (lane 1), a $\Delta invA \Delta spaPQRS \Delta prgHIJK$ mutant derivative (lane 2), or the untagged wild-type strain (lane 3) were separated by BN-PAGE and analyzed by immunoblot for the presence of SpaO or the needle complex. The simultaneous detection of the SpaO (green) and needle complexes (red) from the sample in lane 1 is shown in lane 4. Only the SpaO channel is shown in lanes 1, 2, and 3.

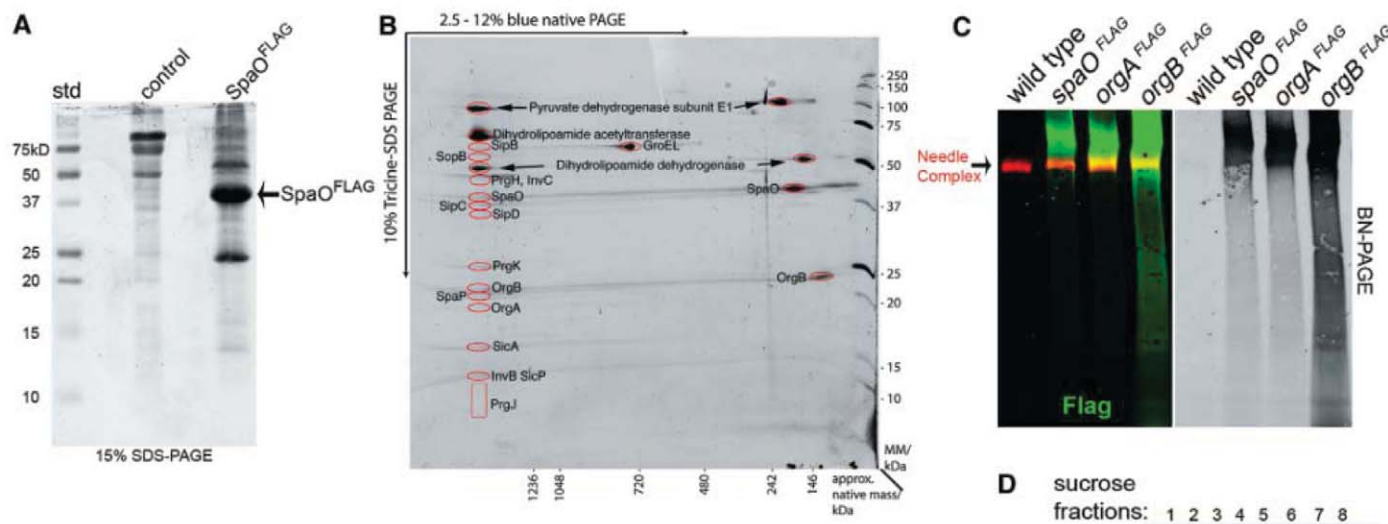
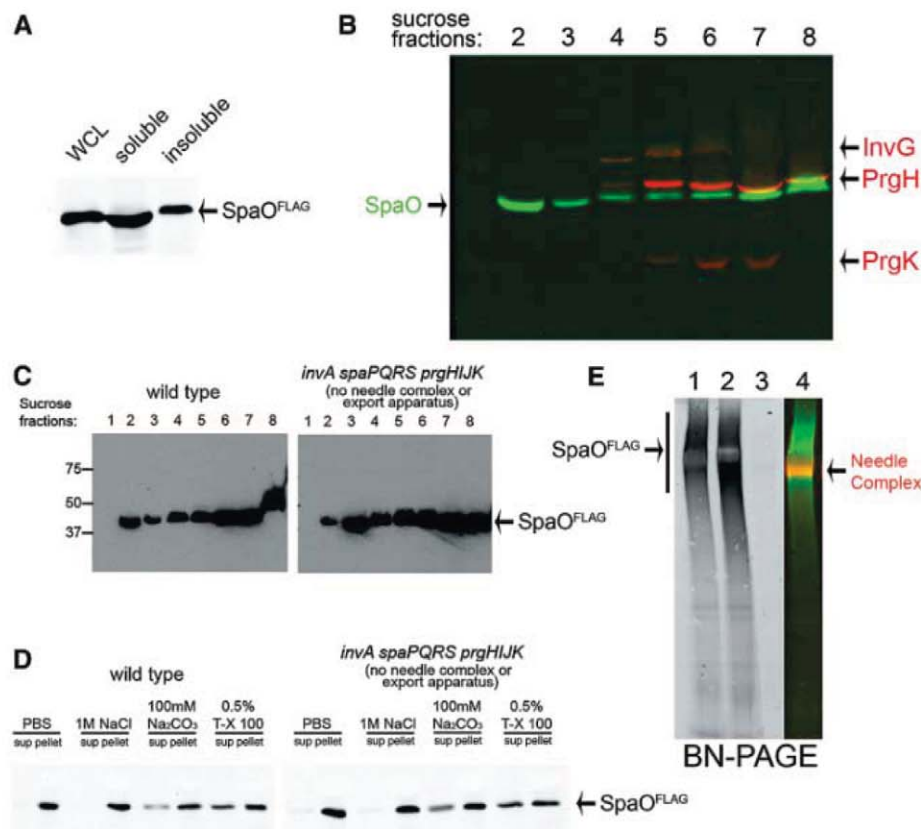
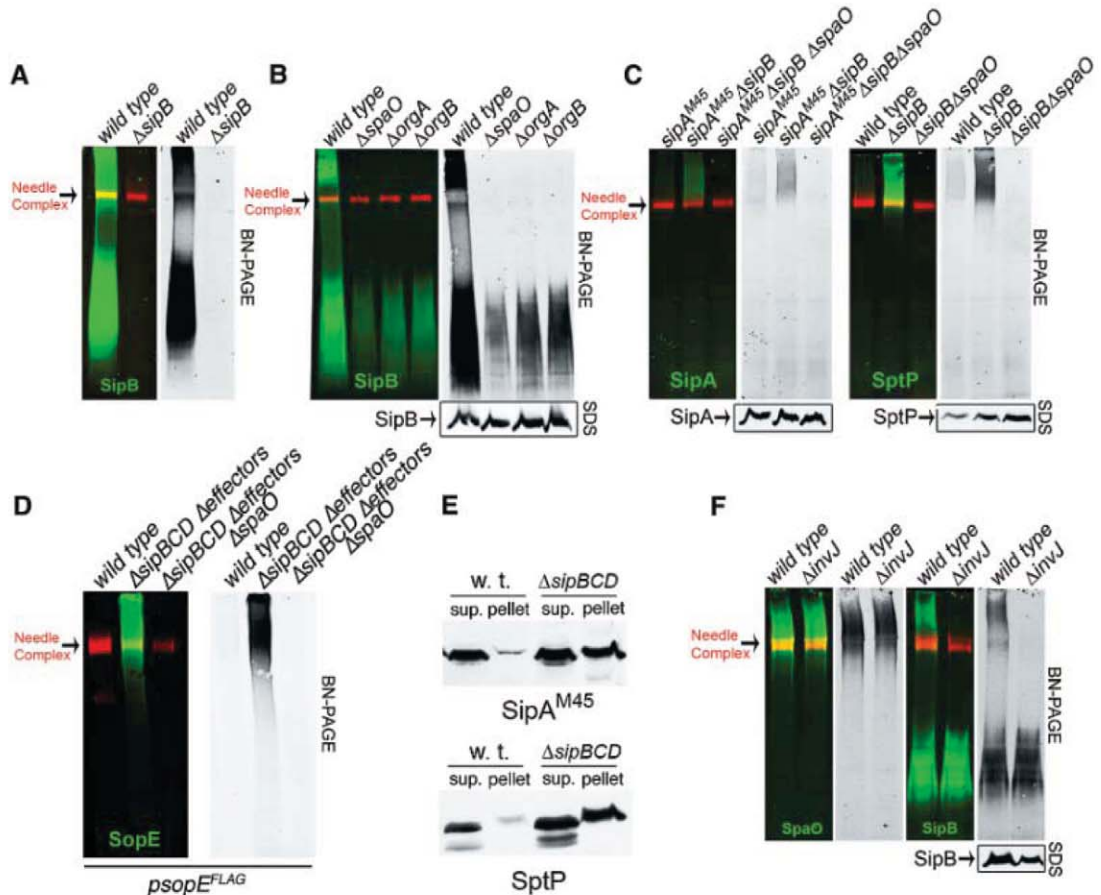


Fig. 2. Characterization of the SpaO complex. (A and B) Cell lysates of wild-type *S. Typhimurium* or an isogenic strain expressing FLAG-epitope-tagged SpaO were immunoprecipitated with an antibody against FLAG and separated by SDS-PAGE (A), and interacting proteins were identified by LC-MS/MS (fig. S1). Alternatively, the pelletable fraction of cell lysates from a *S. Typhimurium*-expressing FLAG-epitope-tagged SpaO was separated on a sucrose density gradient; SpaO-containing fractions were pooled, and SpaO-interacting proteins were immunoprecipitated with an antibody against FLAG and separated by 2D-BN-PAGE (B); and the identity of the proteins in the indicated spots (red circles) was established by LC-MS/MS (fig. S2). (C) Whole-cell lysate of a *S. Typhimurium* strain expressing functional FLAG-epitope-tagged SpaO, OrgA, or OrgB was separated into soluble and pelletable fractions by high-speed centrifugation. Pellet fractions were then applied to a sucrose gradient, and relevant fractions were pooled, separated by BN-PAGE, and analyzed by immunoblot for the presence of SpaO, OrgA, or OrgB (green) or the needle complex (red). The simultaneous detection of the needle complex and SpaO, OrgA, or OrgB is shown in the left (color) panel, and only the detection of SpaO, OrgA, or OrgB is shown in the right (black and white) panel. (D) Subcellular fractionation of OrgA and OrgB. A whole-cell lysate of a *S. Typhimurium* strain expressing FLAG-epitope-tagged OrgA or OrgB was separated into soluble and pelletable fractions by high-speed centrifugation. The pellet fractions were then separated by sucrose gradient centrifugation, and the different gradient fractions were probed for the presence of OrgA or OrgB by immunoblotting.

Fig. 3. The SpaO-OrgA-OrgB platform can be alternatively loaded with different substrates of the type III secretion system. (A and B) Whole-cell lysates of wild-type *S. Typhimurium* or the $\Delta sipB$, $\Delta spaO$, $\Delta orgA$, or $\Delta orgB$ mutants were separated into soluble and pelletable fractions by high-speed centrifugation. Pellets were further fractionated by sucrose gradient centrifugation; relevant fractions were then pooled, separated by BN-PAGE, and analyzed by immunoblot for the presence of SipB (green) or the needle complex (red). Left panels show the simultaneous detection of SipB (green) and the needle complex (red), and right (black and white) panels show only the detection of SipB. The lower panel in (B) shows SDS-PAGE immunoblot analysis of SipB in equal amounts of whole-cell lysates of the indicated strains. (C to E) Samples from the indicated strains were separated into soluble (sup.) and pelletable (pellet) fractions by high-speed centrifugation and separated by SDS-PAGE (E). Alternatively, the pellet fraction was further separated on a sucrose gradient, and the relevant fractions were pooled and separated by BN-PAGE [(C) and (D)]. Left panels in (C) and (D) show the simultaneous detection of SipA, SptP (C), or SopE (D) (green) and the needle complexes (red), and right (black and white) panels show only the channel corresponding to the detection of the respective effector proteins. Lower panels in (C) show SDS-PAGE immunoblot analysis of the indicated proteins in equal amounts of whole-cell lysates of the indicated strains [(C) and (D)]. (F) Samples



from wild-type *S. Typhimurium* or a $\Delta invJ$ mutant expressing a FLAG-tagged SpaO were prepared as indicated above and separated by BN-PAGE. The left panels show the simultaneous detection of SpaO or SipB (green) and the needle complexes (red), and the right (black and white) panels shows only the channel corresponding to the detection of SpaO or SipB. The lower panel shows SDS-PAGE immunoblot analysis of SipB in equal amounts of whole-cell lysates of the indicated strains.

and SipB was present in a complex similar in size to the SpaO-OrgA-OrgB complex (Fig. 3A). The SipB high molecular weight complex was absent from strains lacking SpaO, OrgA, or OrgB (Fig. 3B). The *S. Typhimurium* T3SS is activated upon contact with mammalian cells (16, 24), which results in the stimulation of secretion and the delivery of proteins into target cells. The T3SS of bacteria grown under conditions that stimulate its expression is largely “idle” although it is fully assembled, “poised,” and ready for secretion upon contact with target cells (25). The presence of the protein translocases (which must be secreted before the effectors) in the SpaO-OrgA-OrgB complex, coupled to the observation that most effector proteins were largely absent, suggested that this complex might serve as a sorting platform to “queue” the secreted proteins for sequential orderly delivery. If this were the case, absence of the protein translocases would resemble an “activated” system that has deployed the translocases, and effector proteins should be loaded onto the “sorting platform.” We tested this hypothesis by examining the presence of effector proteins in the SpaO-OrgA-OrgB complex

in the absence of protein translocases. BN-PAGE of samples isolated from a translocase-defective *S. Typhimurium* mutant showed much higher abundance of the effector protein SipA and SptP in the high molecular weight complex when compared to the wild type (Fig. 3C). Indeed, expression of the effector protein SopE in a background strain lacking all the translocases and effectors resulted in a higher abundance of SopE in the high molecular weight complex when compared to the wild type (Fig. 3D). Detection of these effector proteins in a high molecular weight complex was dependent on SpaO (Fig. 3, C and D). Furthermore, the proportion of effector proteins present in high-speed centrifugation pellet fractions increased substantially in the $\Delta sipBCD$ mutant strain (Fig. 3E), which is consistent with their increased loading onto the SpaO-OrgA-OrgB platform in the absence of the translocases. Thus, in the absence of the translocases, effector proteins could associate with the SpaO-OrgA-OrgB platform.

SpaO, OrgA, and OrgB are also required for secretion of the proteins that make up the needle and the rod substructure of the needle complex

(26). They are also required for the secretion of the regulatory protein InvJ (10). In the absence of InvJ, the secretion apparatus is locked in a mode that constitutively secretes the needle and inner rod proteins (but not the translocases) (11). We reasoned that in the absence of InvJ, the SpaO-OrgA-OrgB platform should not be loaded with the protein translocases because, in the absence of substrate switching, the translocases would not be “poised” for secretion. Indeed, high molecular weight complexes obtained from a *S. Typhimurium* $\Delta invJ$ mutant lacked the protein translocase SipB (Fig. 3F), although the formation of the SpaO high molecular weight complex was unaffected (Fig. 3F). Thus, the SpaO-OrgA-OrgB complex serves as a sorting platform that establishes the appropriate hierarchy in the secretion process.

We next investigated the mechanisms by which the SpaO-OrgA-OrgB sorting platform is loaded with the appropriate substrates. Most proteins destined to travel the type III secretion pathway are associated with customized cytoplasmic chaperones, which are necessary for their secretion and in some cases their stability within the

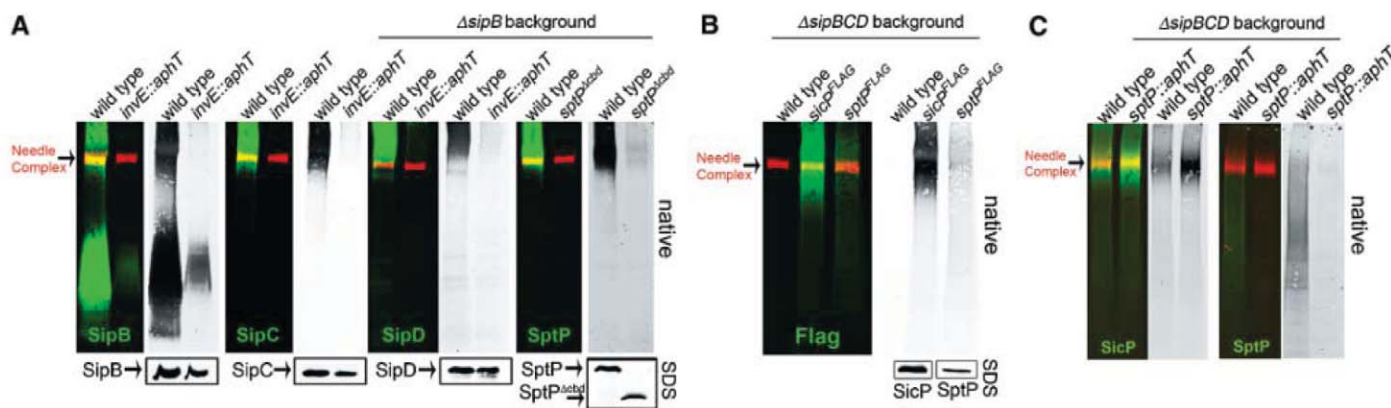


Fig. 4. The type III secretion chaperones are required for the loading of translocases and effector protein onto the SpaO-OrgA-OrgB platform. **(A)** Samples from the wild-type *S. Typhimurium*, a mutant lacking the translocase-chaperone *InvE*, or a strain expressing a mutant of *SptP* lacking its chaperone-binding domain (*SptP*^{Δ35-161}; indicated as *SptP*^{Δcbd}) were prepared as indicated in Fig. 3 and the presence of the translocases *SipB*, *SipC*, and *SipD* and the effector protein *SptP* in the SpaO-OrgA-OrgB complex was examined by BN-PAGE and immunoblot analysis. A *S. Typhimurium* lacking *SipB* was used as a background strain to enhance the detection of *SipD* or *SptP*. In all cases, the left panel shows the simultaneous detection of the indicated effector or translocase (green) and the needle complexes (red) and right (black and white) panels show only the channel corresponding to the detection of the respective effector or translocase proteins. Lower panels show SDS-PAGE immunoblot analysis of the

indicated proteins in equal amounts of whole-cell lysates of the indicated strains. **(B)** Samples from wild-type *S. Typhimurium*, a strain expressing FLAG-epitope-tagged *SicP*, the chaperone for *SptP*, or a strain expressing FLAG-tagged *SptP* were prepared as indicated in Fig. 3 and separated by BN-PAGE. The left panel shows the simultaneous detection of *SicP*^{FLAG} or *SptP*^{FLAG} (green) and the needle complexes (red), and the right (black and white) panel shows only the channel corresponding to the detection of *SicP*^{FLAG} or *SptP*^{FLAG}. The lower panels show SDS-PAGE immunoblot analysis of the indicated proteins in equal amounts of whole-cell lysates of the indicated strains. **(C)** Samples from the indicated strains were prepared as described in Fig. 3 and separated by BN-PAGE. The left panel shows the simultaneous detection of *SicP*^{FLAG} or *SptP* (green) and the needle complexes (red), and the right (black and white) panels show only the channel corresponding to the detection of *SicP*^{FLAG} or *SptP*.

bacterial cytoplasm (27, 28). We tested the potential role of the T3SS-associated chaperones in the recruitment of effectors or translocases to the SpaO-OrgA-OrgB platform. The protein translocases are chaperoned by two proteins, *SicA* and *InvE* (17, 29). In the absence of *InvE*, the translocases *SipB*, *SipC*, or *SipD* were absent from the SpaO-OrgA-OrgB platform, although their total amounts in whole-cell lysates were unchanged (Fig. 4A). Similarly, removal of the chaperone-binding site of *SptP* prevented its recruitment to the SpaO-OrgA-OrgB platform (Fig. 4A), even though the abundance of this effector protein mutant in whole-cell lysates was also unchanged when compared to wild-type *SptP* (Fig. 4A). Thus, the chaperones are required for targeting of the type III secreted proteins to the SpaO-OrgA-OrgB sorting platform. Immediately before secretion, T3SS chaperones are removed from their cognate secreted proteins and remain in the bacterial cytoplasm. We looked for *SicP*, the chaperone of *SptP*, in the SpaO-OrgA-OrgB platform of a *ΔsipBCD* mutant strain (which can be loaded with *SptP*, see above). *SicP* was readily detected in this platform (Fig. 4B), indicating that chaperone removal may occur not upon loading of the SpaO-OrgA-OrgB sorting complex but at a later stage in the secretion process. Furthermore, we detected *SicP* in the SpaO-OrgA-OrgB complex of a *ΔsptP* *S. Typhimurium* mutant strain, which lacks this chaperone's cognate secreted protein *SptP* (Fig. 4C), indicating that recognition of the chaperone-effector complex by the sorting platform may occur through interactions with the chaperone itself.

A distinctive feature of T3SSs is their ability to engage substrates in a pre-established order (9–16).

We have described here a sorting platform (fig. S4) that ensures secretion of the translocases before the effectors. This same platform may also contribute to substrate selection during assembly of the needle complex, although additional mechanisms are required to ensure substrate switching after termination of needle complex assembly (12, 30, 31). Because T3SS-associated chaperones are essential for loading of the sorting platform, the hierarchy of secretion may reflect different affinities of the different secreted protein-chaperone complexes for the sorting platform. The mechanism described here is likely to apply to all T3SSs because the components of the sorting platform and their interactions are conserved in other systems (figs. S5 to S7) (21, 32–35).

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Supporting Online Material

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Materials and Methods

Figs. S1 to S8

Tables S1 and S2

References

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Three-Dimensional Model of *Salmonella*'s Needle Complex at Subnanometer Resolution

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Type III secretion systems (T3SSs) are essential virulence factors used by many Gram-negative bacteria to inject proteins that make eukaryotic host cells accessible to invasion. The T3SS core structure, the needle complex (NC), is a ~3.5 megadalton-sized, oligomeric, membrane-embedded complex. Analyzing cryo-electron microscopy images of top views of NCs or NC substructures from *Salmonella typhimurium* revealed a 24-fold symmetry for the inner rings and a 15-fold symmetry for the outer rings, giving an overall C₃ symmetry. Local refinement and averaging showed the organization of the central core and allowed us to reconstruct a subnanometer composite structure of the NC, which together with confident docking of atomic structures reveal insights into its overall organization and structural requirements during assembly.

Type III secretion systems (T3SSs) are widespread among many pathogenic Gram-negative bacteria such as *Salmonella*, *Yersinia*, *Shigella*, enteropathogenic *Escherichia coli* (EPEC), and *Pseudomonas*. Bacteria deploy the T3SS to transfer bacterial proteins (effectors) into eukaryotic cells. These effectors modulate various cellular functions, which make the host accessible to bacterial invasion (1, 2). The central core element of the T3SS is the needle complex (NC) required to make contact

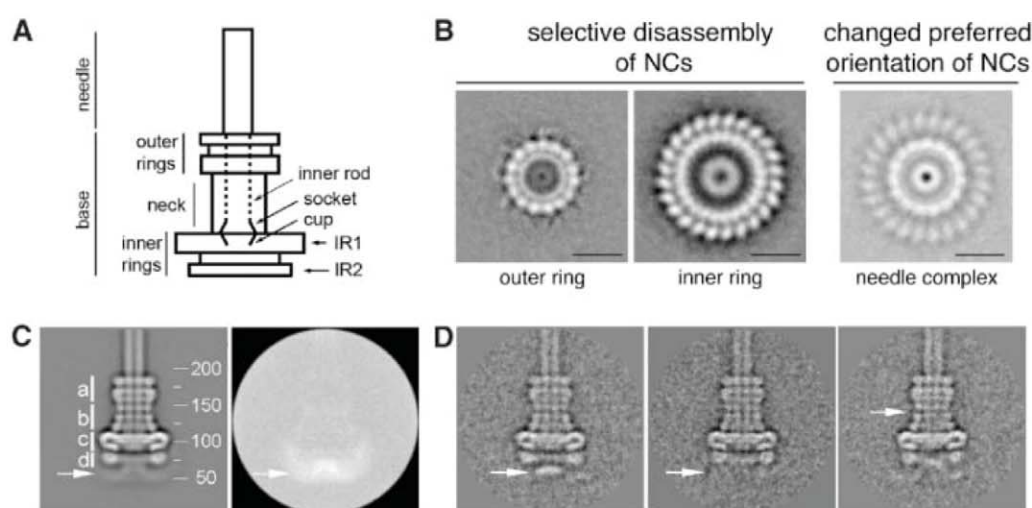
with the host cell (3). It is a large, cylindrically shaped macromolecular complex that is embedded in the inner and outer membrane of bacteria, spans the periplasmic space, and extends into the extracellular environment with a needle filament. Three-dimensional (3D) reconstructions from cryo (4) and negatively stained (5) electron microscopy (EM) images of NCs isolated from *Salmonella* or *Shigella*, respectively, provided the first glimpse of the overall architecture of the core element of the T3SS. In both organisms, NCs are organized as a series of ringlike structures built from individual subunits, suggesting a close structural and evolutionary relationship between different T3SSs and the flagellar system (6–8). Assembly of the ~3.5 MD NC occurs in a stepwise, coordinated

manner, during which multiple copies of the inner and outer membrane proteins organize as rings laterally in their respective membranes to form the base of the NC (Fig. 1A) (9, 10).

Recently, atomic structures of individual domains of major base proteins from various species [PrgH periplasmic domain from *Salmonella* (amino acid 170 to 392), the PrgK homolog EscJ from EPEC (amino acid 21 to 190), and the InvG homolog EcsC from EPEC (amino acid 21 to 174)] became available, and it was speculated that a common structural motif is required for ring formation (11). Moreover, the topology of InvG, PrgH, and PrgK within the NC could finally be directly visualized by EM (12), allowing their rough placement within the complex.

Much less is known about how many copies of the three base proteins are required to build a NC. Strong evidence for 24 subunits comes from the atomic structure of the PrgK homolog EscJ that crystallized as a superhelix with 24 monomers covering one full turn (13) and from the image analysis of projection images obtained from disassembled mutant NCs (12) showing that PrgK builds the smaller of two concentric rings with 24 modulations per turn. The larger ring, which is composed of PrgH in *Salmonella*, also contains 24 subunits, establishing a 1:1 relative stoichiometry (PrgH:PrgK) (12). Similarly, 24 modulations have been observed in top views from *Shigella* NCs (5, 14). Crystal contacts from the InvG homolog of the outer membrane protein EscC from EPEC did not provide any information about subunit number. Nevertheless, for other family members of EscC/InvG (secretin family), 12 to 14 monomers have been discussed to form a ring structure, suggesting that the secretin family has the

Fig. 1. Analysis of symmetry and structural heterogeneity of wild-type NC of *Salmonella typhimurium*. (A) Organization of individual substructures within the NC. (B) (Left and middle) Representative class average of OR and IR substructures after selective NC disassembly and staining with methylamine vanadate. The OR showed 15 repeating subunits (9904 total particles analyzed; 1241 particles/class average) and the IR a concentric ring organization with 24 repeating subunits (1906 total particles analyzed; 928 particles/class average). (Right) Class-average image [$n = 2172$ (total), $n = 224$ (class average)] of top views (cryo-EM images) of intact NC after changing the preferred orientation. For the IR, a 24-fold symmetry (widest diameter) and for the OR, a 15-fold symmetry (small diameter) could be observed. Scale bars, 10 nm. (See figs. S1 and S2.) (C) (Left) Class-average image of cryo-EM images of aligned, nontilted NCs. Letters (a to d) indicate the position of substructures (a, OR; b, neck; c, IR1; d, IR2). The NC is positioned in a square box with dimensions of 256 pixels, and specific pixel levels are indicated with numbers. (Right) Variance analysis



of the nontilted NCs shown in the left panel. Arrow indicates high-variance region below IR2. (D) (Left and middle) Density below IR2 is only seen in some class averages (arrow) and is absent in others. (Right) Class-average image shows a NC with a slightly bent neck (arrow), indicating a flexible connection between the upper and lower neck region.

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potential to adopt multiple symmetries (5, 15–20). In this study, we determined the absolute stoichiometry of PrgH:PrgK:InvG and solved the structure of the NC to subnanometer resolution by cryo-EM. Docking of all three proteins revealed the handedness of the NC and provided insights into its organization at near-atomic resolution.

Although, in principle, analyzing top views of ring-forming complexes is a suitable method to determine their symmetry, the analysis of the T3SS NC is complicated by having two ring structures [inner rings (IR) (PrgH/PrgK) and outer rings (OR) (InvG)] stacked on top of each other (Fig. 1A), resulting in overlapping protein density in EM projections. To unambiguously determine symmetries of the NC inner and outer rings, we therefore aimed to obtain and analyze these structures separately but from previously fully assembled NCs. Using an extended high-pH treatment allowed us to selectively disassemble purified wild-type NCs from *Salmonella* into IR and OR structures (Fig. 1B and fig. S1A). We negatively stained such IR and OR structures using methylamine vanadate (pH 8) and analyzed EM images of top views using multivariate statistical analysis (eigen image analysis, fig. S1B). During the analyses, no symmetry operations were performed. The

IR showed a concentric ring organization with 24 subunits at the two largest rings. This is essentially the same organization as previously described for the less stable mutant complex (PrgHA4), which lacks four amino acids at the C terminus of PrgH (12). The OR clearly showed 15 circularly arranged subunits, suggesting that, together with the 24 subunits present in the inner membrane ring formed by PrgH/PrgK, the entire NC of *Salmonella* displays an overall C3 symmetry. To investigate whether other symmetries can be found in intact NCs, we analyzed top views of fully assembled NCs by cryo-EM. For this, we changed the typically preferred side-view orientation of the NC particles to a top-view orientation by protonation of a poly-histidine tag fused to the N terminus of PrgH. The analysis showed exclusively class averages of NCs with an overall C3 symmetry, composed of a larger ring of 24 and a smaller ring of 15 subunits (Fig. 1B and fig. S2A).

For reconstituting the 3D structure of the NC, we subsequently collected >37,000 particles from vitrified samples. Previous studies described variations in NC diameter and in the structure of the cytoplasmic localized IR2 (4, 5), which is composed of the N-terminal domain of PrgH and the C-terminal domain of PrgK (12). Such variations, which might re-

flect different conformational states, structural flexibilities, or additionally bound proteins, would need to be considered for a 3D reconstruction because they would influence the overall achievable resolution. We therefore studied the structural heterogeneity of individual parts of the NC by single-particle analysis: From the entire particle data set (tilted and nontilted), we used multivariate statistical analysis to generate reference-free class averages and determined their tilt angle by projection matching. To decrease the complexity of analysis and interpretation, only members of nontilted class averages were subsequently analyzed for their variance (Fig. 1, C and D; fig. S4; and movie S1). We found that the most rigid part of the NC is composed of the IR1, the periplasmic localized domain of the IR, and the connecting part of the neck (between levels 80 and 135) (Fig. 1C). The most flexible part of the core NC was at IR2 and the connection between the outer ring and the neck (levels 136 to 200). The greatest variance was observed below IR2, where unidentified additional density was seen in some class averages. The flexibility of IR2 has also been interpreted as an “open” and “closed” conformation (5) and was speculated to be required for the reprogramming of the NC in *Salmonella* (4). Due to the strong variance, we excluded the areas of IR2 and below from subsequent alignments (fig. S3) but kept the data during the final 3D reconstruction.

We reconstructed the structure of the NC (in C1) from ~37,000 cryo-EM images: For the structure determination, two major substructures (IR1/lower neck and OR/upper neck) were first treated separately to recover structural details at higher resolution, and the resulting 3D volumes were then combined into a composite structure. During this procedure, no symmetry operations were applied (image data processing details provided in supporting online material). Subsequently, a threefold symmetrization allowed us to obtain a structure of the *Salmonella typhimurium* NC to ~10 Å (Fig. 2). Clearly, newly discernible features are visible at all levels, in particular details in the individual subunits of the 24-fold IR1 ring and the 15-fold OR/neck. The top-view surface representation shows the organization of the 15- and the 24-fold rings in which five of the OR/neck subunits cover eight of the IR1 subunits (Fig. 2C). The structure also revealed details about the interior elements; in particular, cutting away a vertical segment showed the organization of the socket into individual subunits that directly connect to the inner rod (Fig. 2A). At the cytoplasmic side, the cup, which is believed to be the entry point for substrates, is also separated into individual subunits that can be viewed from the bottom (Fig. 2C). This organization raises the possibility that, upon intimate host cell contact, the delivery of effectors could be triggered because

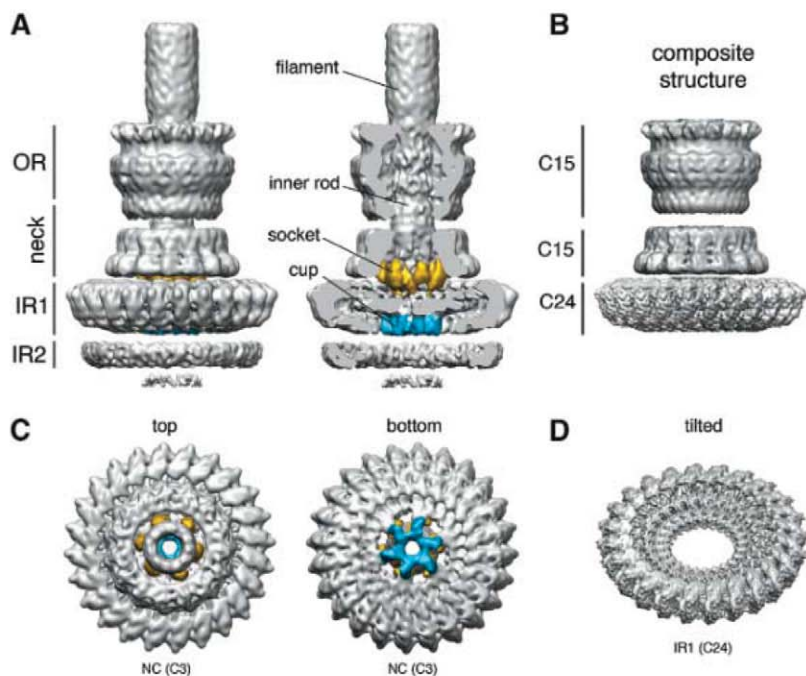


Fig. 2. Three-dimensional surface views of *Salmonella typhimurium*'s NC. (A and C) Surface views of NC 3D reconstruction with C3 symmetry applied and filtered to a resolution of ~10 Å. Side, cut-away (A), top and bottom (C) view show the exterior and interior subunit organization (IR2 removed in bottom view for better visualization of IR1 and cup). The most probable configuration of cup and socket is displayed with respective regions highlighted in blue and yellow (see image data processing details in supporting online material). (B) Applying symmetries to substructures of different components of the NC, that is, a 24-fold for the IR1 and a 15-fold for the neck and OR, led to higher resolutions. (D) Tilted view (45°) of IR1 after symmetrization (C24) revealed structural details to subnanometer resolution.

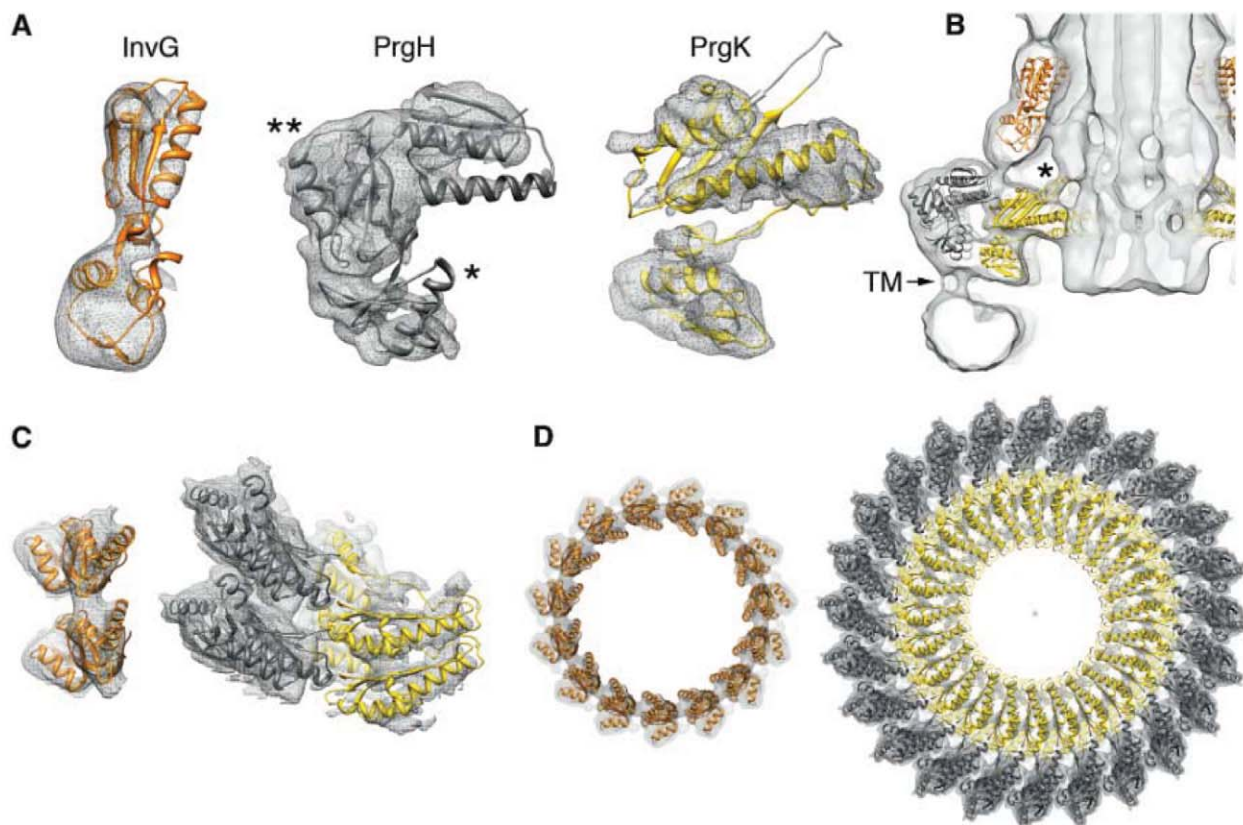


Fig. 3. Docking of atomic structures into the 3D cryo-EM map of the NC provides near-atomic models of the IR1 and the neck region. **(A)** InvG (orange), PrgH (gray), and PrgK (yellow) were independently docked as rigid bodies guided by their domain structure and secondary elements. Tubelike densities, which correspond to α helices, are clearly visible. (For a 3D representation, see also movies S2 to S4.) Positions of amino acids within PrgH are indicated: 267 and 268 (**), and 201 to 206 (*). **(B)** A slice through the NC (side view) with docked atomic structures shows the organization of PrgH and PrgK within IR1 and demonstrates that the cup/socket region is not formed by

these proteins. The asterisk marks a density that connects the modeled PrgK loop with the cup/socket structure. The arrow marks the position of two tubelike densities that most probably correspond to the transmembrane helices (TM) of PrgH and PrgK. **(C)** Magnified top view of neighboring monomers in their respective NC positions. (Left) Organization of two InvG monomers. (Right) Organization of two PrgH and two PrgK monomers. **(D)** 15 InvG (left) and 24 PrgH/PrgK (right) monomers were docked into the lower neck ring and IR1 structure, respectively. PrgH and PrgK are organized as concentric rings with different diameters.

of conformational changes in the needle filament (21–23) that are directly transmitted via the inner rod and socket to the cytoplasmically located cup. Subsequent local symmetrization of IR1 (24-fold), lower neck (15-fold), and OR/upper neck (15-fold) from the 3D structure reconstructed in C1 allowed us to reconstruct substructures at subnanometer resolutions (Fig. 2, B and D, and fig. S5).

In agreement with the recently determined topology of PrgH, PrgK, and InvG (12), we were able to unequivocally dock all available atomic structures of the base proteins into the cryo-electron density map (atomic models for PrgK and InvG were generated from their respective homologous structures, using EscJ and EscC as templates) (fig. S6) and to determine not only the arrangement of individual monomers within their respective rings but also their organization between rings (Fig. 3). (For details, see figs. S8 to S11 and movies S2 to S4.) In the resulting model, all three base proteins build up individual rings and are independently docked as rigid bodies guided by their two-

domain structure and their secondary structural elements: 15 monomers of the N-terminal domain of InvG (orange) are arranged such that the loop containing lysine-38 is located in the vicinity of IR1. This is in agreement with a chemical cross-link found between lysine-38 of InvG and lysine-367 of PrgH (12, 24) (fig. S11). Within the IR1, 24 monomers of PrgH (gray) and PrgK (yellow) were independently docked into the larger and the smaller of the two concentric rings, respectively. The docked PrgH also conforms to the accessibility of an internal his-tag (between amino acid 267 and 268; ** in Fig. 3A) introduced for nano-gold labeling (12). A six-amino acid fragment (201 to 206; * in Fig. 3A; helix 1 in fig. S7) close to the N-terminal part of the periplasmic domain extends out of the electron density map, suggesting that this domain is relaxed when crystallized as an individual protein outside the complex. This could occur as a result of the absence of the connecting transmembrane and cytoplasmic domain of PrgH (Fig. 3B). PrgK is positioned such that its lipidated N-terminal

domain is able to interact with the outer leaflet of the inner membrane (fig. S11). The subsequent domain, however, reaches vertically further up into the complex. From there, PrgK continues with ~60 amino acids, for which no atomic structure is available as of yet, and folds back from its periplasmic location through the membrane toward the cytoplasm (12). This second transmembrane segment is visible in the EM map (Fig. 3B and fig. S11). Furthermore, in PrgK amino acids 135 to 146, which are not present in its homolog EscJ, are modeled as a beta-hairpin (fig. S6) that extends toward the socket. An electron density could be visualized in the corresponding region at elevated density levels (* in Fig. 3B). Taken together, we were able to generate a molecular model of the major part of the NC base (Fig. 4A and movie S5) and established the absolute stoichiometry of PrgH:PrgK:InvG = 24:24:15.

To validate the docking, we tested whether mutations at the PrgH-PrgH interaction surface have an influence on the function of the NC. Our model predicts that amino acid Y239

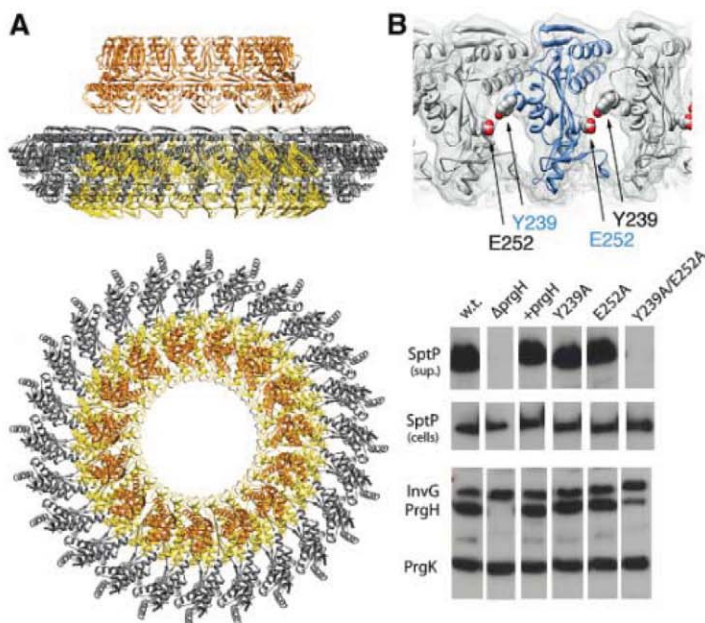


Fig. 4. Atomic model of the NC base and validation of PrgH organization. **(A)** Side and top view of atomic structures of InvG (orange), PrgH (gray), and PrgK (yellow) organized as rings with 15/24/24 subunits, respectively (for a 3D model, see movie S5). **(B)** Point mutations at the predicted interface between PrgH monomers prevent effector protein (SptP) secretion. E252 and Y239 are located at the interaction surface (side chains displayed as spheres). Single-point mutations to alanine showed wild-type level secretion of SptP (sup., supernatant), whereas a double mutant (Y239A/E252A) did not secrete SptP. Cellular SptP levels (cells) in all strains were unchanged. In the PrgH (Y239A/E252A) strain, PrgH protein levels were decreased, indicating that the assembly of NC is compromised, leading to a nonsecreting phenotype. w.t., wild type; $\Delta prgH$ mutant, negative control; $\Delta prgH$, $\Delta prgH$ mutant complemented with $prgH$ -containing plasmid; Y239A and E252, single-point mutated, and Y239A/E252A double-mutated $prgH$ -containing plasmid complementing the $\Delta prgH$ mutant.

in one PrgH monomer is located in the vicinity of amino acid E252 of a second PrgH monomer when assembled as a ring structure (Fig. 4B). Single-point mutations to alanine were not sufficient to change the secretion behavior for the effector protein SptP, indicating that the interaction surface is not sufficiently disturbed and that the individual mutations did not affect the correct folding. The point mutations are located at opposing sites of one monomer, and it thus seems likely that they act independently and that proper folding of PrgH would also not be affected in a double mutant. Yet the level of PrgH is decreased in Y239A/E252A, and no SptP secretion could be detected. Taken together, this suggests that the double mutant does not properly assemble a ring structure and that free PrgH is degraded rapidly, indicating that shortly after expression, wild-type PrgH forms a ring complex and is therefore protected from further degradation.

We showed that the three proteins PrgH, PrgK, and InvG, which dictate the overall shape of the NC base, organize as individual rings of 24 (PrgH/K) and 15 (InvG) subunits and that, as a consequence, the highly oligomeric *S. typhimurium* NC in fact assembles into a complex with an overall low symmetry (C3).

This implies that a local symmetry mismatch exists, in which five of the OR/neck subunits are positioned over eight IR1 subunits. To accommodate this symmetry mismatch, the binding interface must have some structural tolerance that could be important for an efficient assembly when the IR and OR dock onto each other. Structural tolerance could be provided by a weakly ordered interaction surface, which overall could also grant the flexibility needed for the reprogramming phase. Indeed, such a flexibility is supported by the crystallographic analysis of the C-terminal domain of PrgH, in which the last 30 amino acids (363 to 392) could not be resolved (11).

For the cytoplasmically localized ring (IR2), the extensive structural deviations might reflect different conformational or even compositional states and could suggest that IR2 in fact plays an additional functional role during secretion, for example, as a binding platform for a putative C ring (10, 25).

This study provides an experimentally validated near-atomic model of the majority of the NC organelle, and it shows how its central core is organized and engages with the inner rings. Because of the high degree of conservation of T3SSs among pathogens, this structure could be the basis for the understanding of the design,

assembly, and biology of this important translocation machinery and may also pave the way for the development of therapeutic drugs against microbial infections.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/331/6021/1192/DC1
Materials and Methods

Figs. S1 to S17

References

Movies S1 to S5

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Structures of SAS-6 Suggest Its Organization in Centrioles

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Centrioles are cylindrical, ninefold symmetrical structures with peripheral triplet microtubules strictly required to template cilia and flagella. The highly conserved protein SAS-6 constitutes the center of the cartwheel assembly that scaffolds centrioles early in their biogenesis. We determined the x-ray structure of the amino-terminal domain of SAS-6 from zebrafish, and we show that recombinant SAS-6 self-associates in vitro into assemblies that resemble cartwheel centers. Point mutations are consistent with the notion that centriole formation in vivo depends on the interactions that define the self-assemblies observed here. Thus, these interactions are probably essential to the structural organization of cartwheel centers.

Centrioles are ninefold symmetric, cylinder-shaped structures found in most animal cells. They template the nine microtubule doublets in cilia and flagella and constitute the core of the centrosome—the dominant microtubule organizing center in animal cells [reviewed in (1)]. Many diseases like ciliopathies are associated with functional abnormalities in these structures [reviewed in (2)]. In most organisms, immature centrioles (called procentrioles) con-

sist of short microtubule triplets and a structure called the cartwheel, which is made up of a central ringlike hub and nine spokes radiating from the hub [reviewed in (1, 3, 4)] (Fig. 1A). Cartwheel formation occurs at a very early stage of centriole assembly, followed by formation of the peripheral microtubules (5–7). Thus, the cartwheel might serve as a scaffold that determines centriole diameter and symmetry through the radial arrangement of its nine spokes. This idea

is supported by experiments with the two known cartwheel components SAS-6 and CEP135/Bld10. CEP135/Bld10 localizes to the distal part of the cartwheel spokes (Fig. 1A) (8–10) and is essential for centriole formation (8–11). Its truncation in *Chlamydomonas* results in aberrant centrioles with nine shortened cartwheel spokes and a decreased diameter that can accommodate only eight triplet microtubules (9). SAS-6, the other known cartwheel component, is also crucial for centriole assembly (10–22), but localizes to the cartwheel hub (10, 12) and is required for hub formation (10, 12). Lack of SAS-6 in *Chlamydomonas* (12), *Drosophila* (14), and *Paramecium* (10) results in the formation of centrioles with an aberrant number of triplet microtubules, leading to the hypothesis that SAS-6 might self-

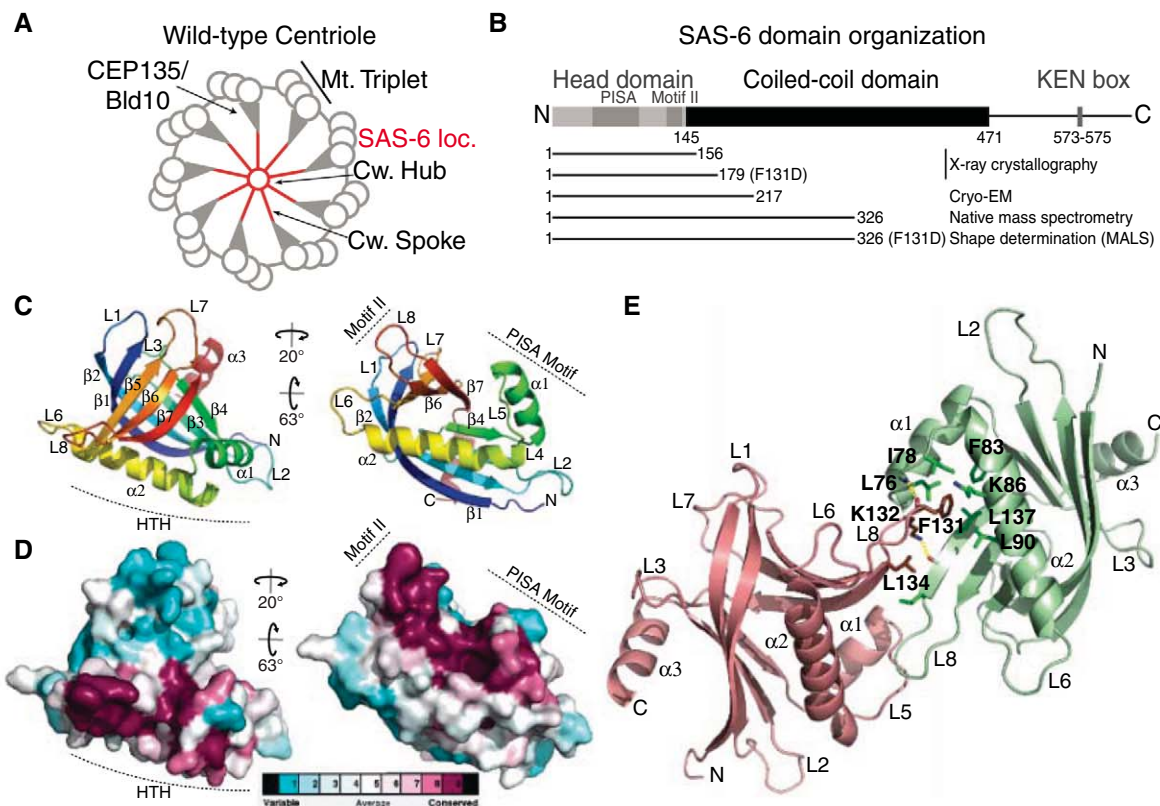
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Fig. 1. Structure and head-to-head dimerization of the N-terminal domain of SAS-6. (A) Overview of the in vivo localization of SAS-6. WT centriole scheme with peripheral microtubule (Mt.) triplets and central cartwheel structure (Cw.) with hub and spokes. The location of SAS-6 is shown in red (12). The localization of CEP135/Bld10 (8, 9) is indicated by an arrow. (B) Domain organization of *D. rerio* SAS-6. The used constructs and the type of experiment performed with them are indicated underneath. KEN box, recognition motif involved in the cell-cycle-dependent degradation of SAS-6 (21); MALS, multi-angular light scattering. (C) Structure of the N-terminal domain of SAS-6 shown in a ribbon diagram and rainbow-colored from N (blue) to C terminus (red). α helices (α), β sheets (β), and loops (L) are numbered sequentially. $\alpha 3$ corresponds to the start regions of the predicted coiled-coil domain in SAS-6. (D) Same as in (C), but as molecular surface with projected sequence conservation denoted by colors ranging from light blue (variable) to burgundy (conserved). The conservation pattern was calculated with ConSurf (35) using a Bayesian method for computing the conservation scores. The



color scale represents the conservation scores. (E) Ribbon presentation of the observed head-to-head dimer of the N-terminal domain of SAS-6 with details of the dimerization interface. The hydrophobic F131 residue inserts into a hydrophobic pocket. Residues that make contact are labeled and shown in sticks for one of the two equivalent interface regions. Hydrogen bonds are indicated by yellow dashed lines.

assemble to form the cartwheel hub and thereby dictate centriole symmetry (3). The molecular structure of cartwheel hubs and SAS-6 and how they direct centriole symmetry is unknown.

SAS-6 consists of a conserved N-terminal domain, a central coiled-coil domain, and a less conserved C-terminal region (Fig. 1B). Because soluble recombinant full-length SAS-6 could not be obtained in sufficient quantities, we determined the three-dimensional structure of the N-terminal domain of *Danio rerio* (zebrafish, Dr) SAS-6 (N-SAS-6¹⁻¹⁵⁶) at 1.92 Å by x-ray crystallography (Fig. 1, B and C, and tables S1 and S2) (23). The asymmetric unit comprises four N-SAS-6¹⁻¹⁵⁶ molecules that superimpose well (fig. S1A). The N-terminal domain of SAS-6 adopts a seven-stranded β -barrel structure capped by two α helices ($\alpha 1$ and $\alpha 2$) at one end that form a helix-turn-helix-like (HTH) motif. The C-terminal helix ($\alpha 3$) that corresponds to the start region of the predicted coiled-coil domain of SAS-6 docks on the barrel wall and makes several polar and hydrophobic contacts with the barrel residues. Notably, despite the absence of sequence similarity, the structure of N-SAS-6¹⁻¹⁵⁶ shows structural similarity to the N-terminal domains of XRCC4 and XLF, both implicated in DNA repair by nonhomologous end-joining (fig. S1B) (24–27).

The N-terminal domain of SAS-6 contains two highly conserved regions (fig. S2) (28): (i) the PISA-motif (18) and (ii) a second motif (motif II) that forms the β hairpin $\beta 6$ – $\beta 7$ that includes the protruding loop 8 (Fig. 1, C and D). The two motifs constitute a continuous highly conserved patch on the N-SAS-6 surface (Fig. 1D and fig. S1, C to E). In the crystal, two N-SAS-6 domains interact to form a head-to-head dimer through this patch (Fig. 1E; see fig. S1, F and G, for a stacking interaction between head-to-head dimers

in the crystal). In this head-to-head dimer, F131 in loop 8 from each monomer is inserted into a hydrophobic pocket on its binding partner formed by the conserved L76, I78, F83, L90, and L137 residues (29) and the aliphatic side chain of K86 (Fig. 1E). The dimer is further stabilized by a hydrophobic contact between residues L134 from both subunits. Additionally, K132 and the main-chain carbonyl group of F131 form hydrogen bonds with the main-chain groups of Q74/T135 and L77, respectively. The area buried on dimer formation is ~ 1500 Å², making dimerization a candidate for a biologically relevant interaction (30, 31).

Analytical gel filtrations with the recombinant N-terminal domains of *D. rerio* (N-SAS-6¹⁻¹⁵⁶) and *Homo sapiens* (N-SAS-6¹⁻¹⁶⁴) suggested possible dimerization of this domain in solution. These proteins eluted at lower molecular sizes when their hydrophobic F131 residue was replaced by aspartic acid, which should weaken head-to-head dimerization (fig. S3, A and B). Analytical ultracentrifugation with *H. sapiens* N-SAS-6¹⁻¹⁶⁴ and *D. rerio* N-SAS-6¹⁻¹⁵⁶ showed that the F131→D131 (F131D) mutants were monomeric, whereas the wild-type (WT) proteins were present in a monomer-dimer equilibrium with apparent dissociation constants of 50 μ M (*Homo sapiens*) and 90 μ M (*Danio rerio*) (Fig. 2A and fig. S3C). These results are consistent with a low-affinity, head-to-head dimerization in solution.

When transiently overexpressed in human U2OS cells, human full-length WT SAS-6 localizes to centrosomes (Fig. 2B) (18) and cytoplasmic foci (18). In contrast, the corresponding SAS-6 F131D mutant was found to be dispersed in the cytoplasm and localized less efficiently to centrosomes (Fig. 2B). This was also observed with the L76D or I78E (V78E in human) mutants that

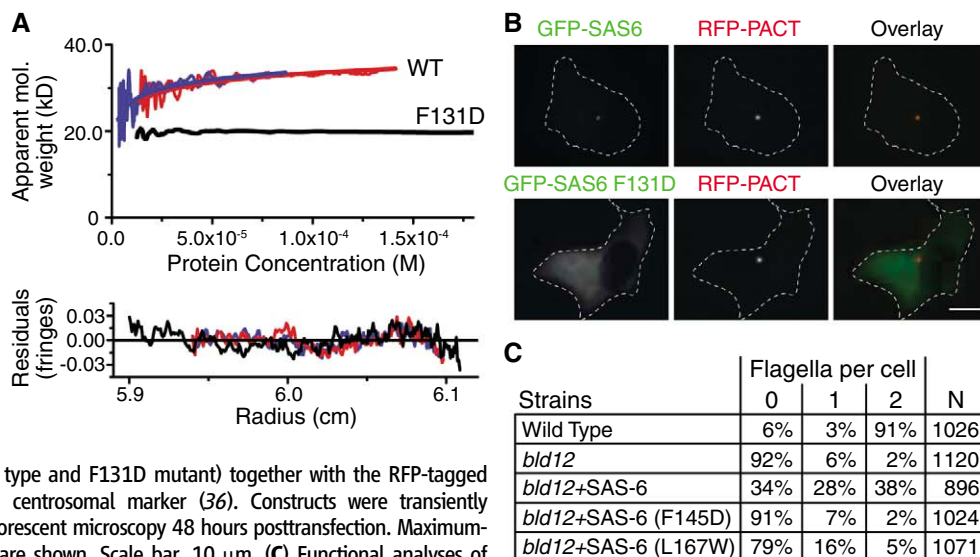
are located in a short loop region of the hydrophobic pocket, into which F131 inserts (fig. S4, B and C).

We tested the *Chlamydomonas* equivalent of the F131D mutant (F145D) (fig. S2) for its functional effect on centriole formation in *Chlamydomonas reinhardtii*. This organism has two flagella that are templated by the two matured centrioles found in each cell. Thus, problems in centriole formation are revealed by aberrant flagellar formation. Because a null mutant of SAS-6, *bld12*, has severe defects in centriole formation, $\sim 90\%$ of cells lack flagella (Fig. 2C) (12). When transformed with a cDNA construct coding for full-length WT *Chlamydomonas* SAS-6, the percentage of flagellated *bld12* cells increased to 66%; in contrast, the F145D mutant of SAS-6 showed no recovery in flagellar formation (Fig. 2C), although SAS-6 expression levels were comparable (fig. S5).

The similarity between SAS-6, XLF, and XRCC4 suggests that SAS-6, like XLF (24, 25) and XRCC4 (26, 27), could form a parallel coiled-coil dimer. To avoid potential complications in crystal packing due to the presence of two dimerization interfaces, we used the F131D mutation to disrupt the head-to-head dimerization of SAS-6. We determined the crystal structure of Dr N-SAS-6¹⁻¹⁷⁹ F131D at 1.98 Å (Fig. 3, A and B, and table S2). Two of the three molecules present in the asymmetric unit (fig. S6, A and B) formed a parallel coiled-coil dimer (Fig. 3A) through a canonical knob-into-holes packing of hydrophobic residues of the coiled-coil stalks that is further stabilized by a side-chain-to-side-chain H-bonding network between D146, T147, and K150 and a salt bridge between E163 and K164 (Fig. 3B and fig. S6C). Consistent with the structural model, light scattering measurements with Dr N-SAS-6¹⁻³²⁶ F131D,

Fig. 2. Head-to-head dimerization of SAS-6

occurs in solution and is crucial for centriole formation. (A) Sedimentation equilibrium analysis of the molecular weight of human N-SAS-6¹⁻¹⁶⁴ in solution (calculated molecular weight = 19.8 kD). (Top) Apparent molecular weight as a function of concentration. Replicates of the WT protein (blue and red traces) and the F131D mutant (black trace) are shown. The smooth traces were produced by a regularization algorithm implemented in the UltraSpin software. (Bottom) Residuals of the fit of the interference data to single- (for the F131D mutant) and double-component (for the wild type) models. (B) Projected fluorescence microscopy images of human U2OS cells expressing N-terminally green fluorescent protein-tagged full-length human SAS6 (wild type and F131D mutant) together with the RFP-tagged C-terminal PACT domain of pericentrin as a centrosomal marker (36). Constructs were transiently transfected into U2OS cells and analyzed by fluorescent microscopy 48 hours posttransfection. Maximum-intensity projected images of transfected cells are shown. Scale bar, 10 μ m. (C) Functional analyses of mutated SAS-6 using a *Chlamydomonas* Δ SAS-6 mutant. The C-terminally haemagglutinin (HA) epitope-tagged WT and mutated SAS-6 genes were introduced into the *bld12-1* mutant, and clones expressing each protein were isolated from the transformants. The table shows the percentages of those cells in each clone with zero, one, or two flagella. The percentages of flagellated cells in the *bld12* strain transformed with WT SAS-6 are lower than those in the WT *Chlamydomonas* strain. This may be due to the presence of the HA tag in these constructs. N, number of cells counted.



which contains ~56% of the predicted coiled-coil domain of SAS-6, suggest that SAS-6 stably dimerizes in solution through an elongated coiled-coil domain (fig. S7).

Removal of ~90% of the predicted coiled-coil region of human SAS-6 (SAS-6 Δ 167-466)

led to its strong mislocalization in U2OS cells (fig. S4D). We also disturbed the coiled coil of SAS-6 and its packing against the head domains (fig. S6D) by introducing a bulky tryptophan residue at the L153 equivalent (L167W) in *Chlamydomonas* SAS-6 and assayed its effect on the

ability to rescue the *bld12* phenotype. As shown in Fig. 2C, expression of the mutant SAS-6 slightly increased the number of flagellated cells (21%), but the percentage was much less than that of the clone transformed with WT SAS-6, although SAS-6 expression levels were comparable (fig. S5). Circular dichroism spectroscopy results suggest that the L153W mutation does not disturb the fold of SAS-6 (fig. S8).

A SAS-6 construct containing both dimerization interfaces forms higher-order oligomers in solution, as judged by native mass spectrometry (fig. S9). Our structural models suggest that these oligomers may be curved (Fig. 4A). A purified construct containing both interfaces (Dr N-SAS-6¹⁻²¹⁷) crystallized spontaneously in solution, giving rise to small, low-diffraction quality crystals. Cryo-electron microscopy (cryo-EM) images (Fig. 4B) of these crystals revealed notable ring-shaped (or, less likely, spiral-shaped) assemblies. These assemblies contain 16 distinct, slightly oval blobs that seem to stack on each other, resulting in long tubes connected by radially projecting spokes. The lengths of the spokes are consistent with the predicted coiled-coil length (~10.5 nm) of Dr N-SAS-6¹⁻²¹⁷. The diameter of these assemblies is ~217 Å, as measured from the center of one oval blob to an equivalent position across the ring center.

The modeled N-SAS-6¹⁻¹⁷⁹ tetramer shown in Fig. 4A overlays well with four of these blobs (Fig. 4B). Tube formation may be based on stacking interactions between the head-to-head dimers, similar to those that we observed in the N-SAS-6¹⁻¹⁵⁶ crystal (fig. S1F). In our overlay in Fig. 4B, the coiled-

Fig. 3. SAS-6 forms a parallel coiled-coil dimer. (A) Structure of the N-SAS-6¹⁻¹⁷⁹ F131D dimer drawn in ribbon presentation with subunits shown in green and red. (B) Detailed view of the dimer interface. The dimer is stabilized by a canonical hydrophobic packing (some of the involved residues are labeled and shown as sticks); a H-bonding network between D146, T147, and K150; and a salt bridge between E163 and K164 (dashed yellow lines).

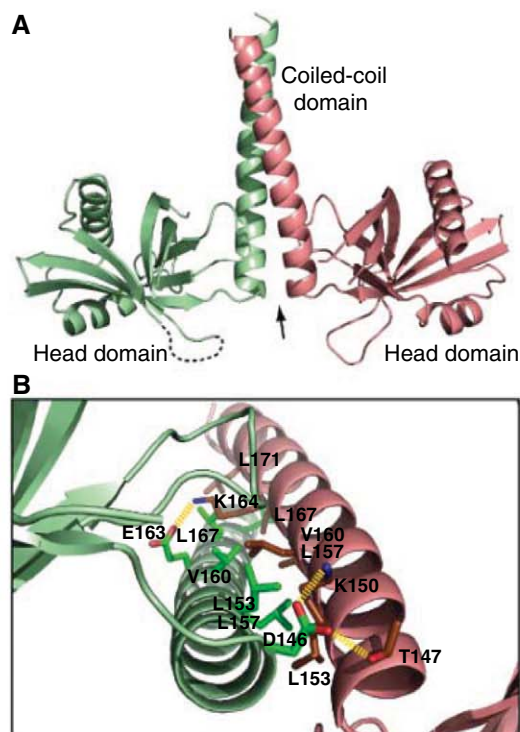
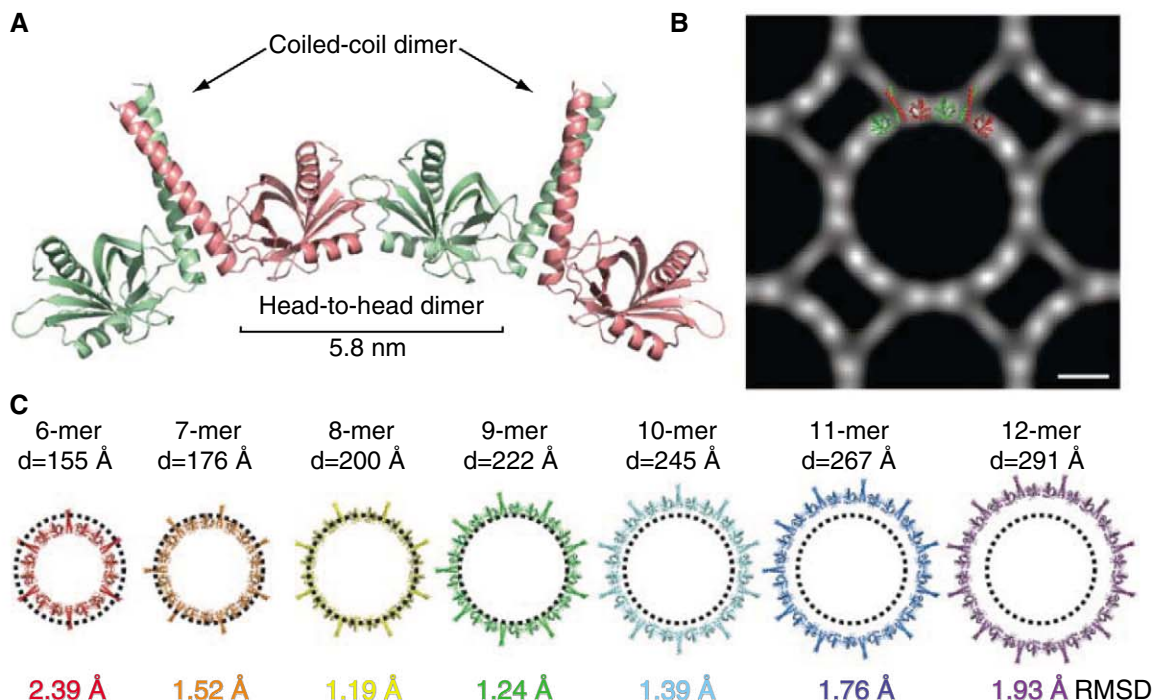


Fig. 4. Model of SAS-6 ring assembly. (A) Ribbon presentation of a modeled SAS-6 tetramer based on the observed coiled-coil and head-to-head dimers. The distance between the base regions of the two coiled-coil domains is indicated. (B) Cryo-EM image of a face-on view of a thin crystal of N-SAS-6¹⁻²¹⁷. Pixel size, 3.74 Å per pixel; scale bar, 60 Å. The image was Fourier-filtered and symmetry-averaged. The SAS-6 tetramer presented in (A) is shown as an overlay. The overlaid structure is based on N-SAS-6¹⁻¹⁷⁹, which has a shorter coiled coil than N-SAS-6¹⁻²¹⁷. (C) Models of SAS-6 rings with different symmetries. The approximate diameters of these rings (the double distance from head-domain center to ring center) are indicated above the modeled rings. The diameter of cartwheel hubs observed in procenterioles by cryo-electron tomography (32) is shown as dotted circles. To model these rings, we allowed for a change in the orientation of the head domains relative to the coiled-



coil domain. To compare the required changes, we calculated the root mean square deviation (RMSD) between the N-SAS-6¹⁻¹⁷⁹ structure and two equivalent head domains in the modeled ring. These values are indicated under the modeled rings.

coil domain in the crystal structure would need to be at an angle to better fit the EM density. This could be accommodated by some flexibility between the head domains and the coiled-coil domain of SAS-6. Allowing for this flexibility, we modeled ring assemblies containing 6 to 12 SAS-6 dimers (Fig. 4C). Compared with the N-SAS-6¹⁻¹⁷⁹ F131D crystal structure, the eight- and ninefold symmetric rings required the smallest changes in the orientation of the head domains. With their radially projecting, stalklike coiled-coil domains, these ring assemblies resemble centriolar cartwheels. The inner diameter of the modeled ninefold symmetric ring was comparable to the diameter of the cartwheel hubs observed in procentrioles by cryo-electron tomography (200 Å) (32). Consistent with our ring models with projecting coiled-coil domains, the C-termini of SAS-6 in *Chlamydomonas* are found in the outer regions of the centriolar cartwheel spokes (fig. S10).

No cartwheel has been identified so far in *Caenorhabditis elegans*. Instead, a central centriolar tube was found, whose presence requires SAS-6 (33). This alternative assembly could be due to structural differences in SAS-6, as motif II in *C. elegans* and *C. briggsae* is very distinct from the other SAS-6 homologs [fig. S2 and (28)].

We suggest that SAS-6 self-assembly is a contributing factor in the structural organization of centriolar cartwheels cores. However, our data also suggest that other centriolar components are probably needed for a faithful and stable ninefold symmetric SAS-6 assembly in vivo. This notion is in agreement with the presence of alternative SAS-6 assemblies that are observed when SAS-6 is overexpressed in *Drosophila* (14, 34).

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Supporting Online Material

www.sciencemag.org/cgi/content/full/science.1199325/DC1

Materials and Methods

Figs. S1 to S10

Tables S1 and S2

References

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DAXX/ATRX, MEN1, and mTOR Pathway Genes Are Frequently Altered in Pancreatic Neuroendocrine Tumors

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Pancreatic neuroendocrine tumors (PanNETs) are a rare but clinically important form of pancreatic neoplasia. To explore the genetic basis of PanNETs, we determined the exomic sequences of 10 nonfamilial PanNETs and then screened the most commonly mutated genes in 58 additional PanNETs. The most frequently mutated genes specify proteins implicated in chromatin remodeling: 44% of the tumors had somatic inactivating mutations in *MEN1*, which encodes menin, a component of a histone methyltransferase complex, and 43% had mutations in genes encoding either of the two subunits of a transcription/chromatin remodeling complex consisting of DAXX (death-domain-associated protein) and ATRX (α thalassemia/mental retardation syndrome X-linked). Clinically, mutations in the *MEN1* and *DAXX/ATRX* genes were associated with better prognosis. We also found mutations in genes in the mTOR (mammalian target of rapamycin) pathway in 14% of the tumors, a finding that could potentially be used to stratify patients for treatment with mTOR inhibitors.

Pancreatic neuroendocrine tumors (PanNETs) are the second-most common malignancy of the pancreas. The 10-year survival rate

of patients with PanNETs is only 40% (1–3). PanNETs are usually sporadic but can arise in multiple endocrine neoplasia type 1 and more

rarely in other syndromes, including von Hippel-Lindau (VHL) syndrome and tuberous sclerosis (4). “Functional” PanNETs secrete hormones that cause systemic effects, whereas “nonfunctional” PanNETs do not and therefore cannot always be readily distinguished from other neoplasms of the pancreas. Nonfunctional PanNETs grow silently, and patients often present with either an asymptomatic abdominal mass or symptoms of abdominal pain secondary to compression by a large tumor. Surgical resection is the treatment of choice, but many patients present with unresect-

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able tumors or extensive metastatic disease, and medical therapies are relatively ineffective in these cases.

There is currently insufficient information about this tumor to either predict prognosis of patients diagnosed with PanNETs or to develop companion diagnostics and personalized treatments to improve disease management. Biallelic inactivation of the *MEN1* gene, usually through a mutation in one allele coupled with

loss of the remaining wild-type allele, occurs in 25 to 30% of PanNETs (5, 6). *MEN1* is a tumor suppressor gene that, when mutated in the germline, predisposes to multiple endocrine neoplasia type 1 syndrome. Chromosomal gains and losses and expression analyses have revealed candidate loci for genes involved in the development of PanNETs, but these have not been substantiated through genetic or functional analyses (7–9).

To gain insights into the genetic basis of this tumor type, we determined the exomic sequence of ~18,000 protein-coding genes in a discovery set of 10 well-characterized sporadic PanNETs. A clinically homogeneous set of tumors of high neoplastic cellularity is essential for the successful identification of genes and pathways involved in any tumor type. Thus, we excluded small-cell and large-cell neuroendocrine carcinomas and studied only samples that were not part of a familial syndrome associated with PanNETs (table S1) (1). We microdissected tumor samples in order to achieve a neoplastic cellularity of >80%. DNA from the enriched neoplastic

samples and from matched non-neoplastic tissue from 10 patients was used to prepare fragment libraries suitable for massively parallel sequencing. The coding sequences were enriched by capture with the SureSelect Enrichment System and sequenced by use of an Illumina GAIIX platform (10). The average coverage of each base in the targeted regions was 101-fold, and 94.8% of the bases were represented by at least 10 reads (table S2).

We identified 157 somatic mutations in 149 genes among the 10 tumors used in the discovery set. The mutations per tumor ranged from 8 to 23, with a mean of 16 (table S3). Of these mutations, 91% were validated by means of Sanger sequencing. There were some obvious differences between the genetic landscapes of PanNETs and those of pancreatic ductal adenocarcinomas (PDAC) (11). First, there were 60% fewer genes mutated per tumor in PanNETs than in PDACs. Second, the genes most commonly affected by mutation in PDACs (*KRAS*, *TGF-β* pathway, *CDKN2A*, and *TP53*) were rarely altered in PanNETs and vice versa (Table 1). Third, the

Table 1. Comparison of commonly mutated genes in PanNETs and PDAC based on 68 PanNETs and 114 PDACs.

Genes*	PanNET	PDAC†
<i>MEN1</i>	44%	0%
<i>DAXX</i> , <i>ATRX</i>	43%	0%
Genes in mTOR pathway	15%	0.80%
<i>TP53</i>	3%	85%
<i>KRAS</i>	0%	100%
<i>CDKN2A</i>	0%	25%
<i>TGFB1</i> , <i>SMAD3</i> , <i>SMAD4</i>	0%	38%

Table 2. Mutations in *MEN1*, *DAXX*, *ATRX*, *PTEN*, *TSC2*, *PIK3CA*, and *TP53* in human PanNETs. (hom) indicates these mutations appear homozygous.

Sample*	Gene	Transcript accession	Nucleotide (genomic)†	Nucleotide (cDNA)	Amino acid (protein)‡	Mutation type
PanNET3PT	<i>ATRX</i>	CCDS14434.1	g.chrX:76716462G>A (hom)	c.6235C>T(hom)	p.R2079X	Nonsense
PanNET5PT	<i>ATRX</i>	CCDS14434.1	g.chrX:76742636G>A	c.5620C>T	p.Q1874X	Nonsense
PanNET13PT	<i>ATRX</i>	CCDS14434.1	g.chrX:76741560delA	c.5932delT	fs	Indel
PanNET27PT	<i>ATRX</i>	CCDS14434.1	g.chrX:76700959_76700962delATAA	c.6338_6341delTTAT	fs	Indel
PanNET35PT	<i>ATRX</i>	CCDS14434.1	g.chrX:76806893_76806909delAA TTTCTTCTAAAAGCA	c.3824_3840delTGG CTTTTGAAGAAATT	fs	Indel
PanNET52PT	<i>ATRX</i>	CCDS14434.1	g.chrX:76796337_76796340delCTTT	c.4221_4224delAAAG	fs	Indel
PanNET59PT	<i>ATRX</i>	CCDS14434.1	g.chrX:76761014C>A	c.5364G>T	p.Q1788H	Missense
PanNET78PT	<i>ATRX</i>	CCDS14434.1	g.chrX:76665406C>T	c.6829G>A	p.E2277K	Missense
PanNET85PT	<i>ATRX</i>	CCDS14434.1	g.chrX:76794404dupC	c.4414dupG	fs	Indel
PanNET98PT	<i>ATRX</i>	CCDS14434.1	g.chrX:76700832T>A(hom)	c.6468A>T(hom)	p.Q2156H	Missense
PanNET100PT	<i>ATRX</i>	CCDS14434.1	g.chrX:76762518_76762521 delCACT(hom)	c.5270_5272delAGTG(hom)	fs	Indel
PanNET112PT	<i>ATRX</i>	CCDS14434.1	g.chrX:76826041T>A(hom)	c.1363A>T(hom)	p.K455X	Nonsense
PanNET25PT	<i>DAXX</i>	CCDS4776.1	g.chr6:33394939delT	c.1976delA	fs	Indel
PanNET31PT	<i>DAXX</i>	CCDS4776.1	g.chr6:33394935delC(hom)	c.1980delG(hom)	fs	Indel
PanNET44PT	<i>DAXX</i>	CCDS4776.1	g.chr6:33394795delG	c.2120delC	fs	Indel
PanNET56PT	<i>DAXX</i>	CCDS4776.1	g.chr6:33397319delG	c.211delC	fs	Indel
PanNET77PT	<i>DAXX</i>	CCDS4776.1	g.chr6:33396614G>A	c.916C>T	p.R306X	Nonsense
PanNET84PT	<i>DAXX</i>	CCDS4776.1	g.chr6:33395309delG	c.1766delC	fs	Indel
PanNET87PT	<i>DAXX</i>	CCDS4776.1	g.chr6:33397141A>C	c.389T>G	p.L130R	Missense
PanNET93PT	<i>DAXX</i>	CCDS4776.1	g.chr6:33396641C>G	c.889G>C	p.A297P	Missense
PanNET94PT	<i>DAXX</i>	CCDS4776.1	g.chr6:33394872_33394873insA	c.2042_2043insT	fs	Indel
PanNET95PT	<i>DAXX</i>	CCDS4776.1	g.chr6:33397221_33397224delCGCC	c.306_309delGGCG	fs	Indel
PanNET96PT	<i>DAXX</i>	CCDS4776.1	g.chr6:33396167delC	c.1219delG	fs	Indel
PanNET97PT	<i>DAXX</i>	CCDS4776.1	g.chr6:33395838C>A(hom)	c.1393G>T(hom)	p.E465X	Nonsense
PanNET102PT	<i>DAXX</i>	CCDS4776.1	g.chr6:33397515T>A	c.166A>T	p.K56X	Nonsense
PanNET103PT	<i>DAXX</i>	CCDS4776.1	g.chr6:33397579delA	c.102delT	fs	Indel
PanNET104PT	<i>DAXX</i>	CCDS4776.1	g.chr6:33396604_33396605insACT(hom)	c.925_926insAGT(hom)	p.L309QF	Indel/missense
PanNET108PT	<i>DAXX</i>	CCDS4776.1	g.chr6:33395828delT(hom)	c.1403delA(hom)	fs	Indel
PanNET133PT	<i>DAXX</i>	CCDS4776.1	g.chr6:33395889C>A(hom)	c.1342G>T(hom)	p.E448X	Nonsense
PanNET3PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64331709C>A(hom)	c.689G>T(hom)	p.G230V	Missense
PanNET5PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64332046A>G(hom)	c.562T>C(hom)	p.W188R	Missense
PanNET6PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64333812_64333828delCA CGGCTGGAGACACCC	c.329_345delGGG TGTTCTCAGCCGTG	fs	Indel

continued on next page

Sample* Sample*	Gene Gene	Transcript access- Transcript accession	Nucleotide (genomic)† Nucleotide (genomic)†	Nucleotide (cDNA) Nucleotide (cDNA)	Amino acid (protein)‡ Amino acid (protein)‡	Mutation Mutation type
PanNET10PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64334105_64334108 delTCGT(hom)	c.50_53delACGA(hom)	fs	Indel
PanNET23PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64331233_64331234delAG(hom)	c.832_833delCT(hom)	fs	Indel
PanNET29PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64334070C>A(hom)	c.88G>T(hom)	p.E30X	Nonsense
PanNET31PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64328587G>T	c.1643C>A	p.S548X	Nonsense
PanNET39PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64333993_64333999 delAGGGATG(hom)	c.159_165delCATCCCT(hom)	fs	Indel
PanNET44PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64333955delG	c.203delC	fs	Indel
PanNET45PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64330370G>C	c.974C>G	p.P325R	Missense
PanNET52PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64333876delG	c.282delC	fs	Indel
PanNET57PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64334002delG(hom)	c.156delC(hom)	fs	Indel
PanNET59PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64329234G>A	c.1213C>T	p.Q405X	Nonsense
PanNET61PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64334049_64334201delG GAGCACCAGTCC GGCTCCTCTCGGCC CAGCTCGGCAGCAAA CAGGGCGACCACGTCGTCGA TGGAGCGCAGCGGGAA CAGCGCTCTTGGGGCGGCC TTCAGCCCCATGGCGGCGGGCGG TGGGCGGCGGCCCTG CAAGGCAAGCCGGGGAG(hom)	c.1_109delAT GGGGCTGAAGCCCG CCCAGAAGACGCTGTT CCCCTGCGCTCCATCGAC GACGTGGTGGCGCTGTTTG CTGCCGAGC TGGGCCGAGAGGA GCCGGACCTGGTGCTCC(hom)	fs	Indel
PanNET64PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64333781delC	c.377delG	fs	Indel
PanNET69PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64330291delA	c.1053delT	fs	Indel
PanNET77PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64334063_64334079delGG CTCCTCTCGGCCAG	c.79_95delCT GGGCCGAGAGGAGCC	fs	Indel
PanNET78PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64332045C>T	c.563G>A	p.W188X	Nonsense
PanNET83PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64330369delG	c.975delC	fs	Indel
PanNET84PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64334139G>A	c.19C>T	p.Q7X	Nonsense
PanNET85PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64332011_64332012insCTGT	c.596_597insACAG	fs	Indel
PanNET93PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64333906_64333909delAGAC	c.249_252delGTCT	fs	Indel
PanNET94PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64333906_64333909delAGAC	c.249_252delGTCT	fs	Indel
PanNET95PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64332032delC(hom)	c.576delG(hom)	fs	Indel
PanNET96PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64331269T>C	c.IVS799-2A>G	c.IVS799-2A>G	Splice site
PanNET99PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64331938C>A	c.IVS669+1G>T	c.IVS669+1G>T	Splice site
PanNET100PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64331940_64331941delCG(hom)	c.667_668delCG(hom)	fs	Indel
PanNET102PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64332102G>A	c.506C>T	p.A169V	Missense
PanNET108PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64331200_64331251delG CAGCCTGGCCAC TTCCCTCTACTGACCTTT CCAGATGTCCCAGGTCATAGA(hom)	c.815_837delTC TATGACCTGGGA CATCTGGAA(hom)	del exon and intron	Indel
PanNET109PT	<i>MEN1</i>	CCDS8083.1	g.chr11:64334093A>C	c.65T>G	p.L22R	Missense
PanNET10PT	<i>PIK3CA</i>	CCDS43171.1	g.chr3:180418785G>A	c.1633G>A	p.E545K	Missense
PanNET10PT	<i>PTEN</i>	CCDS31238.1	g.chr10:89707693delG	c.738delG	fs	Indel
PanNET31PT	<i>PTEN</i>	CCDS31238.1	g.chr10:89682819T>G	c.323T>G	p.L108R	Missense
PanNET29PT	<i>PTEN</i>	CCDS31238.1	g.chr10:89710790_89710791ins TGACAAGGAATAT CTAGTACTACTTTAA	c.961_962insTGA CAAGGAATATCTAG TACTTACTTTAA	fs	Indel
PanNET96PT	<i>PTEN</i>	CCDS31238.1	g.chr10:89675287T>C(hom)	c.202T>C(hom)	p.Y68H	Missense
PanNET104PT	<i>PTEN</i>	CCDS31238.1	g.chr10:89701856G>A(hom)	c.494G>A(hom)	p.G165E	Missense
PanNET24PT	<i>TP53</i>	CCDS11118.1	g.chr17:7518284G>A	c.722C>T	p.S241F	Missense
PanNET91PT	<i>TP53</i>	CCDS11118.1	g.chr17:7519210delA(hom)	c.445delT(hom)	fs	Indel
PanNET100PT	<i>TP53</i>	CCDS11118.1	g.chr17:7520101delT(hom)	c.311delA(hom)	fs	Indel
PanNET2PT	<i>TSC2</i>	CCDS10458.1	g.chr16:2070191C>T	c.3422C>T	p.A1141V	Missense
PanNET31PT	<i>TSC2</i>	CCDS10458.1	g.chr16:2074957G>A	c.4498G>A	p.V1500M	Missense
PanNET44PT	<i>TSC2</i>	CCDS10458.1	g.chr16:2074337_2074338delTG	c.4113_4114delTG	fs	Indel
PanNET70PT	<i>TSC2</i>	CCDS10458.1	g.chr16:2078571C>T	c.5383C>T	p.R1795C	Missense
PanNET93PT	<i>TSC2</i>	CCDS10458.1	g.chr16:2038643C>A	c.26C>A	p.S9X	Nonsense
PanNET112PT	<i>TSC2</i>	CCDS10458.1	g.chr16:2076836A>G	c.4952A>G	p.N1651S	Missense

*Samples PanNET3, PanNET7, PanNET10, PanNET21, PanNET23, PanNET24, PanNET25, PanNET31, PanNET36, and PanNET93 were used for the initial (discovery set) screen. †Coordinates refer to human reference genome hg18 release (NCBI 36.1, March 2006). ‡Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

spectrum of mutations in PDAC and PanNET were different, with C-to-T transitions more common in PDACs than in PanNETs and C-to-G transversions more common in PanNETs than in PDACs (table S4). This suggests that mutations in PanNETs and PDAC arise through different mechanisms, perhaps because of exposure to different environmental carcinogens or through the action of different DNA-repair pathways.

We next selected genes for further analysis that were well-documented components of a pathway that was genetically altered in more than one tumor because alterations in these genes are most likely to be clinically relevant. Four genes were mutated in at least two tumors in the discovery set: *MEN1* in five, *DAXX* in three, *PTEN* in two, and *TSC2* in two. *ATRX* was mutated in only one sample in the discovery set, but its product forms a heterodimer with *DAXX* and, therefore, is part of the same pathway, so it was also evaluated in the validation set. Similarly, *PIK3CA* was included because its product is part of the mammalian target of rapamycin (mTOR) pathway that includes *PTEN* and *TSC2* (12–14). The sequences of these genes were then determined by means of Sanger sequencing in a validation set consisting of 58 additional PanNETs and their corresponding normal tissues (Fig. 1, A and B). In total, somatic muta-

tions in *MEN1*, *DAXX*, *ATRX*, *PTEN*, *TSC2*, and *PIK3CA* were identified in 44.1%, 25%, 17.6%, 7.3%, 8.8%, and 1.4% PanNETs, respectively (Table 2).

Of the 30 mutations in *MEN1*, 25 were inactivating mutations [18 insertions or deletions (“indels”), 5 nonsense, and 2 splice-site mutations], whereas five were missense. At least 11 were homozygous; in the others, the presence of “contaminating” DNA from normal cells made it difficult to reliably distinguish heterozygous from homozygous changes. *MEN1* encodes menin, a nuclear protein that acts as a scaffold to regulate gene transcription by coordinating chromatin remodeling. It is an essential component of the MLL SET1-like histone methyltransferase (HMT) complex (15–19). Overall, *MEN1* was mutated in 30 of the 68 PanNETs used in the discovery and validation sets combined.

DAXX and *ATRX* were mutated in 17 and 12 PanNETs, respectively. No tumor with a mutation in *DAXX* had a mutation in *ATRX*, which is consistent with their presumptive function within the same pathway. Overall, 29 of 68 PanNETs (42.6%) had a mutation in this pathway. There were 11 indels and four nonsense mutations in *DAXX* and six indels and three nonsense mutations in *ATRX*. The three *ATRX* missense mutations were within the conserved helicase

domain, whereas the *DAXX* missense mutations were nonconserved changes. Five *DAXX* and four *ATRX* mutations were homozygous, indicating loss of the other allele. The high ratio of inactivating-to-missense mutations in both genes establishes them as PanNET tumor suppressor genes. Loss of immunolabeling for *DAXX* and *ATRX* correlated with mutation of the respective gene (fig. S1, A and B, and table S5). From these data, we hypothesize that both copies of *DAXX* are generally inactivated, one through mutation and the other either through loss of the nonmutated allele or epigenetic silencing. We also hypothesize that both copies of *ATRX* are inactivated, one through mutation and the other through chromosome X inactivation. Recently, it has been shown that *DAXX* is an H3.3-specific histone chaperone (20). *ATRX* encodes a protein that at the amino-terminus has an ADD (*ATRX*-DNMT3-DNMT3L) domain and a carboxy-terminal helicase domain. Almost all missense disease-causing mutations are within these two domains (21). *DAXX* and *ATRX* interact, and both are required for H3.3 incorporation at the telomeres; *ATRX* is also required for suppression of telomeric repeat-containing RNA expression (22–24). *ATRX* was recently shown to target CpG islands and G-rich tandem repeats (25), which exist close to telomeric regions.

We identified five *PTEN* mutations, two indels and three missense; six *TSC2* mutations, one indel, one nonsense, and four missense; and one *PIK3CA* missense mutation. Previously published expression analyses have indicated that the expression of genes in the mTOR pathway is altered in most PanNETs (26, 27). Our data suggest that, at least at the genetic level, only a subset of PanNETs have alterations of this pathway. This finding may have direct clinical application through prioritization of patients for therapy with mTOR pathway inhibitors. Everolimus [also called Afinitor, RAD-001, and 40-O-(hydroxyethyl)-rapamycin] has been shown to increase progression-free survival in a subset of PanNET patients with advanced disease (28). If the mutational status of genes coding for proteins in the mTOR pathway predicts clinical response to mTOR inhibitors, it should be possible to select patients who would benefit most from an mTOR inhibitor through analysis of these genes in patients’ tumors (29, 30).

All 68 tumors evaluated in this study were from patients undergoing aggressive intervention (table S6) and included patients undergoing curative resection as well as those with metastatic disease. Mutations in *MEN1*, *DAXX/ATRX*, or the combination of both *MEN1* and *DAXX/ATRX* were associated with prolonged survival relative to those patients whose tumors lacked these mutations (Fig. 1, C and D, and table S7). This was particularly evident in patients with metastatic disease and with mutations in both *MEN1* and *DAXX/ATRX*: 100% of patients with PanNETs that had these mutations survived at least 10 years,

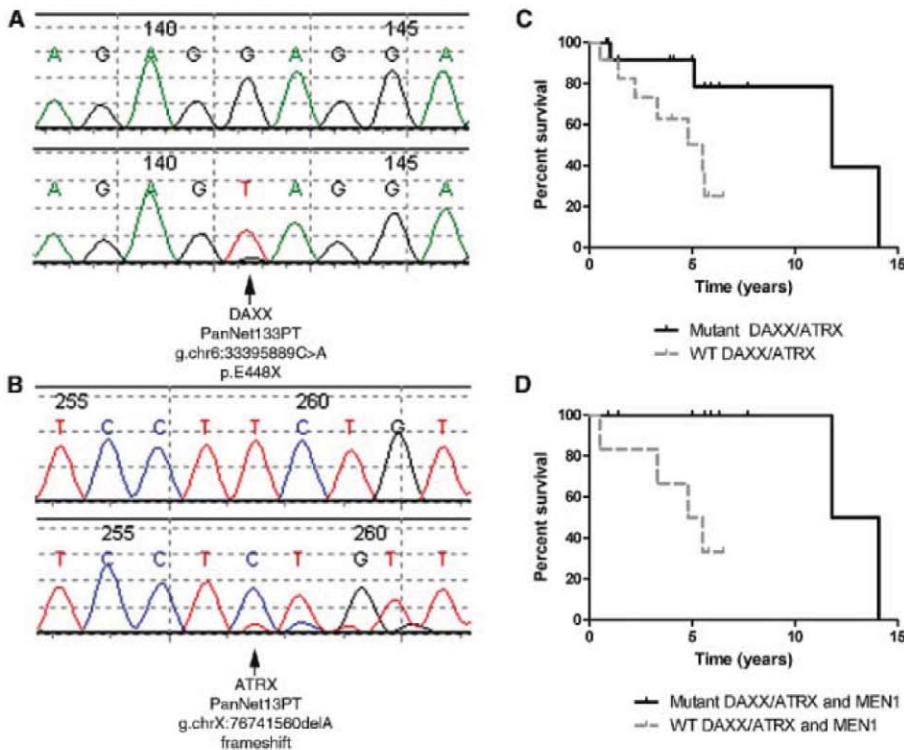


Fig. 1. (A and B) Examples of traces showing mutations in DNA isolated from cancer cells [(A) and (B), bottom] but not from normal cells of the same patient [(A) and (B), top]. (C and D) Kaplan-Meier plots of overall survival of patients with metastatic PanNETs. (C) Fifteen patients with a *DAXX* or *ATRX* gene mutation versus 12 patients in whom both genes were wild type (WT) [hazard ratio 0.22, 95% confidence interval (CI) 0.06 to 0.84, $P = 0.03$]. (D) Nine patients with mutations in *MEN1* as well as either *DAXX* or *ATRX* versus six patients in which all three genes were WT (hazard ratio 0.07, 95% CI 0.009 to 0.53, $P = 0.01$). All types of mutations in these genes were included in the analysis.

whereas over 60% of the patients without these mutations died within 5 years of diagnosis (Fig. 1D). One possible explanation for the difference in survival is that mutations in *MEN1* and *DAXX/ATRX* identify a biologically specific subgroup of PanNETs.

Whole-exome sequencing of pancreatic neuroendocrine tumors has led to the identification of previously unknown tumor suppressor genes and illuminated the genetic differences between the two major neoplasms of the pancreas. The mutations may serve to aid prognosis and provide a way to prioritize patients for therapy with mTOR inhibitors.

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Supporting Online Material

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Materials and Methods

Fig. S1

Tables S1 to S8

References

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Different B Cell Populations Mediate Early and Late Memory During an Endogenous Immune Response

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Memory B cells formed in response to microbial antigens provide immunity to later infections; however, the inability to detect rare endogenous antigen-specific cells limits current understanding of this process. Using an antigen-based technique to enrich these cells, we found that immunization with a model protein generated B memory cells that expressed isotype-switched immunoglobulins (swlg) or retained IgM. The more numerous IgM⁺ cells were longer lived than the swlg⁺ cells. However, swlg⁺ memory cells dominated the secondary response because of the capacity to become activated in the presence of neutralizing serum immunoglobulin. Thus, we propose that memory relies on swlg⁺ cells until they disappear and serum immunoglobulin falls to a low level, in which case memory resides with durable IgM⁺ reserves.

Memory B cells are generated during the primary immune response to foreign antigens. This process is initiated when naïve B cells expressing surface immunoglobulin (Ig) bind the antigen in secondary lymphoid organs, receive signals from helper T cells, and proliferate (1). This proliferation produces short-lived immunoglobulin-secreting plasmablasts and germinal center cells, many of which switch their immunoglobulin constant region from IgM to IgG, IgA, or IgE and acquire somatic mutations

in the variable region (1–3). Cells that acquire immunoglobulin mutations that improve antigen binding gain a survival advantage and emerge from the germinal center reaction as long-lived surface-switched immunoglobulin (swIg)⁺ memory cells, or surface Ig⁺ plasma cells that maintain serum immunoglobulin levels (4). After subsequent exposure to antigen, the memory cells proliferate rapidly and generate plasmablasts, which boost the amount of antigen-specific immunoglobulin in the serum to aid in antigen clearance

(1, 4). There is, however, evidence for the existence of IgM⁺ memory B cells that have or have not passed through germinal centers or undergone somatic mutation (5).

Recently, genetic labeling of B cells that expressed activation-induced cytidine deaminase (AID), which is required for isotype switching and somatic mutation (6), suggested that IgM⁺ memory cells make up part of the memory B cell pool in mice (7). Whether these cells were antigen-specific was not addressed. Thus, the relative contribution of IgM⁺ B cells—especially those that may not express AID—to the antigen-specific memory pool remains unclear.

We sought to gain a comprehensive view of all memory B cells in normal mice by tracing the fate of antigen-specific precursors throughout the primary immune response on the basis of antigen-specificity alone without the complications related to the use of immunoglobulin transgenic mice (8–10). Phycoerythrin (PE) and allophycocyanin were chosen as model foreign antigens because their fluorescent properties

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allowed direct flow cytometric detection of B cells expressing complementary immunoglobulin (11, 12). Naïve PE-specific B cells could not, however, be detected in a conventional 10^6 -cell sample of the 2×10^8 spleen and lymph node cells from a mouse that had never been exposed to PE (Fig. 1, A and B). To solve this problem, antigen-specific B cells from the entire spleen and lymph node cell sample were enriched with magnetic beads (13). Naïve PE-specific B cells, mainly of the $CD43^- CD21^- CD23^+$ B2 phenotype (fig. S1A) were detected among the cells in a sample that bound to a magnetic column after staining with PE and magnetic beads coated with antibody against PE (Fig. 1C). The unbound cells generated a PE-specific antibody response when transferred into B cell-deficient hosts that was only 20% that of unfractionated spleen and lymph nodes, which suggested that about 80% of the naïve PE-specific B cell population was captured by the enrichment procedure (fig. S1B). The PE-specific B cells that were missed may have had immunoglobulin that bound PE with very low affinity. The enrichment approach revealed that naïve B6 mice contained ~20,000 PE-specific B cells (Fig. 1D) in the spleen and lymph nodes. In contrast, naïve mice contained only 4000 B cells specific for allophycocyanin (Fig. 1D), which indicated that preimmune populations specific for different antigens vary in size. PE-specific cells were not detected in PE-enriched samples from MD4 transgenic mice (14) that contain only monoclonal hen egg lysozyme-specific B cells (Fig. 1E), which demonstrated the specificity of the enrichment method.

PE-specific B cells increased dramatically in the draining lymph nodes of B6 mice after subcutaneous injection of PE in complete Freund's adjuvant (CFA) but not after CFA alone (Fig. 1, F and G). Note that our gating strategy excluded the $B220^-$, PE^{low} non-B cells (Fig. 1F) that were capable of capturing secreted immunoglobulins (15) (fig. S1C). The PE-specific B cells peaked at $\sim 10^6$ cells by day 13 then declined to a stable population of $\sim 150,000$ cells (Fig. 1G).

$CD38$, $GL7$, and immunoglobulin isotype switching were monitored to assess the cell types present in the PE-specific B cell population (16, 17). PE-specific B cells in naïve mice expressed IgM and $CD38$ but not $GL7$ and contained low amounts of intracellular immunoglobulin, as expected for naïve cells (Fig. 2, A and C). In contrast, the PE-specific population present 8 days after PE and CFA injection contained subsets that expressed IgM or swIg (Fig. 2D), which could be divided further into intracellular Ig^{high} plasmablasts (Fig. 2, E and G), $CD38^- GL7^+$ germinal center cells, and $CD38^+ GL7^-$ naïve or memory cells (Fig. 2, F and H).

These populations underwent dramatic changes over time. PE-specific plasmablasts, both IgM^+ and sw Ig^+ , peaked at $\sim 20,000$ cells between days 10 and 13, as PE-specific immunoglobulin appeared in the serum, and then declined rapidly

by day 30 (Fig. 2I). IgM^+ and sw Ig^+ PE-specific germinal center cells peaked on day 13 at 400,000 cells, and declined slowly out to day 100 (Fig. 2J), probably because of the slow release of PE from the CFA emulsion (18).

$CD38^+ GL7^-$ PE-specific sw Ig^+ memory cells were first detected on day 6, peaked on day 30 at $\sim 60,000$ cells, and declined to very low numbers by day 450 (Fig. 2K). By day 7, $CD38^+ GL7^-$ PE-specific IgM^+ cells began to increase over naïve levels, peaked on day 10 at $\sim 120,000$ cells (Fig. 2K), and remained remarkably stable until at least day 450. To confirm that the $CD38^+ GL7^-$ PE-specific IgM^+ cells in primed mice were genuine memory cells, we labeled IgM^+ B cells from $CD45.1^+$ naïve mice with carboxyfluorescein succinimidyl ester (CFSE) and transferred them into naïve $CD45.2^+$ hosts to track their division history. Nine days after PE and CFA injection, 75% of the $CD45.1^+ CD38^+ IgM^+$ PE-specific B cells (Fig. 3, A and B) had lost CFSE (Fig. 3C) but were no longer blasts (Fig. 3D). Thus, both populations fit the definition of memory cells (19), having responded to antigenic stimulation, retained $CD38$ expression, and become quiescent. The difference in the life-span of the populations could not be explained by differential expression of the survival-promoting tumor ne-

crosis factor receptor superfamily member 13c (BAFFR) (20) (fig. S2A). sw Ig^+ cells, however, had higher amounts of tumor necrosis factor receptor superfamily member 13b (TACI), which may inhibit B cell survival (21).

Both sw Ig^+ and IgM^+ PE-specific memory cells were T cell-dependent (fig. S3). The populations differed, however, in that IgM^+ memory cells retained IgD, expressed lower amounts of memory cell surface markers (22) (fig. S2B), and contained fewer mutations in complementarity-determining regions (CDRs) 1 and 2 of the heavy chain (Fig. 3E, and tables S1 and S2) than sw Ig^+ memory cells. In accordance with the latter finding, sw Ig^+ , but not the IgM^+ memory cell population, underwent affinity maturation, manifested by increased PE binding capacity, until the germinal center reaction peaked (Fig. 3F). Thus, the IgM^+ memory cells showed less evidence of germinal center selection, affinity maturation, and differentiation than sw Ig^+ memory cells.

Immunized mice were then challenged with an intraperitoneal injection of PE to assess memory B cell function. Mice that were primed with PE 320 days earlier and contained 100,000 PE-specific IgM^+ and 2000 sw Ig^+ memory cells produced 25,000 sw Ig^+ plasmablasts but very few IgM^+ plasmablasts or germinal center cells of either type

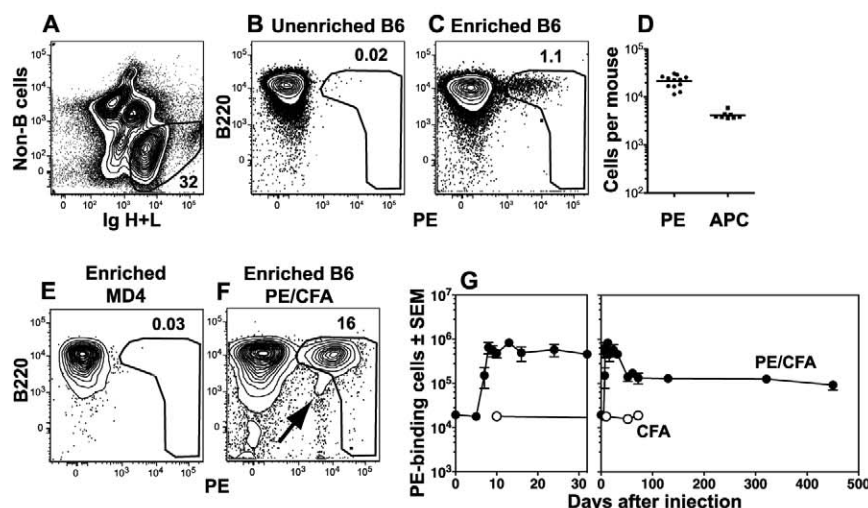


Fig. 1. Detection of PE-specific B cells. (A) B cells were identified by flow cytometry in spleen and lymph node samples as cells that did not bind a cocktail of antibodies specific for $CD4$, $CD8$, $CD11c$, $Gr1$, or $F480$ (non-B cells) and expressed immunoglobulin heavy and light chains (H+L, both intracellular and extracellular). The cells with large amounts of immunoglobulin were plasmablasts. (B) Representative flow cytometric analysis of unenriched spleen and lymph node B cells from a naïve B6 mouse after staining with PE. (C) Representative flow cytometric analysis of spleen and lymph node B cells from a naïve B6 mouse after staining with PE and enrichment with anti-PE magnetic beads. (D) Number of PE- or allophycocyanin (APC)-specific B cells in individual naïve B6 mice. The bar indicates the mean. (E and F) Representative flow cytometric analysis of spleen and lymph node B cells from (E) a naïve MD4 $Rag1^{-/-}$ mouse or (F) a B6 mouse, 24 days after subcutaneous injection of 15 μ g PE and CFA, that were stained with PE and enriched with anti-PE magnetic beads. The PE gate was drawn to exclude cells that bind antigen-antibody complexes [arrow in (F)]. (G) Combined data from multiple experiments showing the numbers of PE-specific B cells in the spleen and lymph nodes at the indicated times after subcutaneous injection of 15 μ g PE and CFA (filled circles) or CFA alone (open circles) shown over the first 32 (left) or 450 (right) days. The mean and SEM ($n = 3$ to 6) are shown for all time points except the day 61 time point for PE and CFA-injected mice (where $n = 2$, and the mean is shown without error bars) and the day 52 and day 72 time points for mice injected with CFA alone ($n = 1$) (10).

(Fig. 4A). The number of swIg⁺ memory cells increased 150-fold over the prechallenge level, whereas the number of IgM⁺ memory cells increased less than twofold (Fig. 4A). Thus, antigen

restimulation caused swIg⁺ memory cells to generate plasmablasts and more memory cells without new germinal center cells, whereas IgM⁺ memory cells responded poorly.

One possibility, however, was that IgM⁺ memory cells switched immunoglobulin isotype after challenge and contributed to the swIg⁺ progeny. This possibility was difficult to assess as

Fig. 2. Detection of PE-specific plasmablasts, germinal center cells, and memory B cells. PE-specific cells enriched from the spleen and lymph nodes of (A to C) a naïve B6 mouse or (D to H) a B6 mouse on day 8 after subcutaneous injection of PE and CFA, identified as shown in Fig. 1. (A and D) Flow cytometry-based gating strategy used to identify cells expressing surface IgM and/or surface and intracellular IgG1, IgG2c, IgG2b, IgG3, IgA, and IgE. (B, E, and G) Flow cytometry-based gating strategy used to identify Ig^{low} nonplasmablasts (left) or Ig^{high} plasmablasts (PBs) (right) within the IgM⁺ or swIg⁺ populations. (C, F, and H) Flow cytometry-based gating strategy used to identify CD38⁺ GL7[−] naïve and/or memory (N/M) or CD38[−] GL7⁺ germinal center (GC) cells within the non-PB populations. (I to K) Combined data from multiple experiments showing the numbers of IgM⁺ (open symbols, solid line) or swIg⁺ (filled symbols, solid line) PE-binding plasmablasts (I), germinal center cells (J), or memory cells (K) in the spleen and lymph nodes after subcutaneous injection of PE and CFA on day 0. The mean and

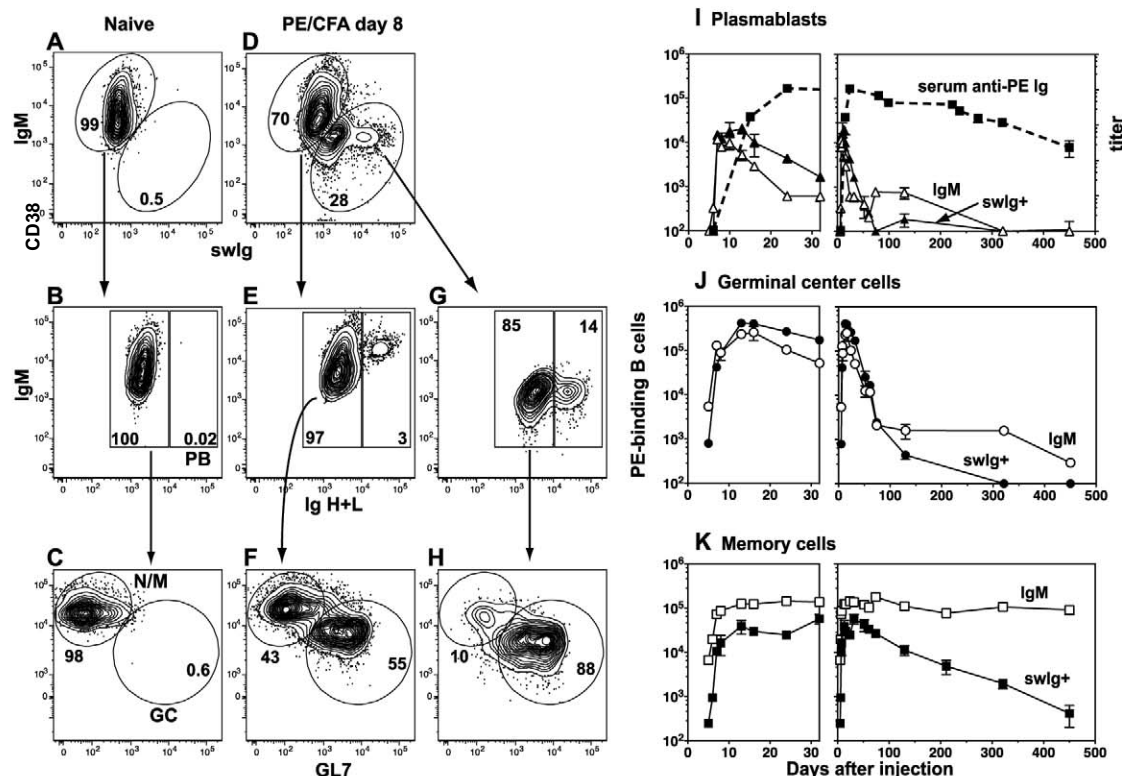
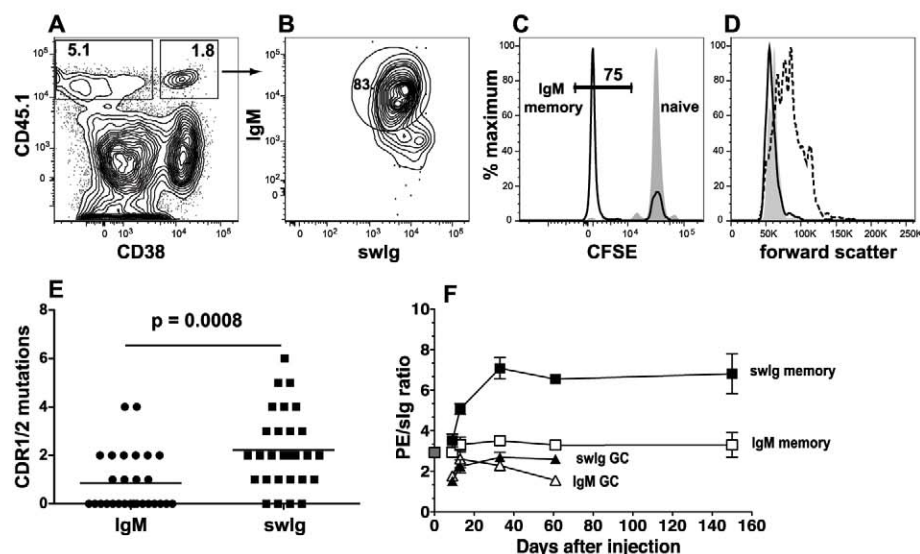


Fig. 3. Properties of the PE-specific IgM⁺ memory B cell population. (A to D) PE-specific cells enriched from B6 recipients of CD45.1⁺ CFSE-labeled B cells, 9 days after subcutaneous injection of PE and CFA or CFA alone, identified as in Fig. 1. (A) Flow cytometry-based gating strategy used to identify CD45.1⁺ donor PE-specific B cells that did (right) or did not (left) express CD38. (B) Flow cytometry-based gating strategy used to identify CD38⁺, CD45.1⁺ PE-specific B cells that expressed IgM. (C) Flow cytometric analysis of CFSE in CD45.1⁺ PE-specific CD38⁺ IgM⁺ cells from mice injected with CFA alone (filled) or PE and CFA (black line). The frequency of CFSE^{low} cells is indicated for PE and CFA-injected mice. (D) Representative flow cytometric analysis of forward light scatter of PE-specific IgM⁺ CD38⁺ naïve cells from mice injected with CFA alone (filled), or PE-specific CD38⁺ IgM⁺ memory cells (black line), or CD38[−] IgM⁺ cells from mice injected with PE and CFA (dashed line). (E) The number of mutations resulting in amino acid substitutions, in the CDR1 and two variable region heavy chain genes for each of 28 PE-binding IgM⁺ or IgG1⁺ clones from mice injected 25 days earlier with PE and CFA. Statistical significance was established with an unpaired Student's *t* test. (F) Combined data from two independent experiments showing the ratio of PE fluorescence intensity divided by total immunoglobulin fluorescence intensity for naïve (gray filled



square) IgM⁺ memory (open squares), swIg⁺ memory (black filled squares), IgM⁺ germinal center (open triangles), or swIg⁺ germinal center (black filled triangles) cells at the indicated times after PE and CFA injection. The mean and SEM (n = 3 to 4) are shown for all time points except day 0 and day 61, where n = 2, and the mean is shown without error bars (10).

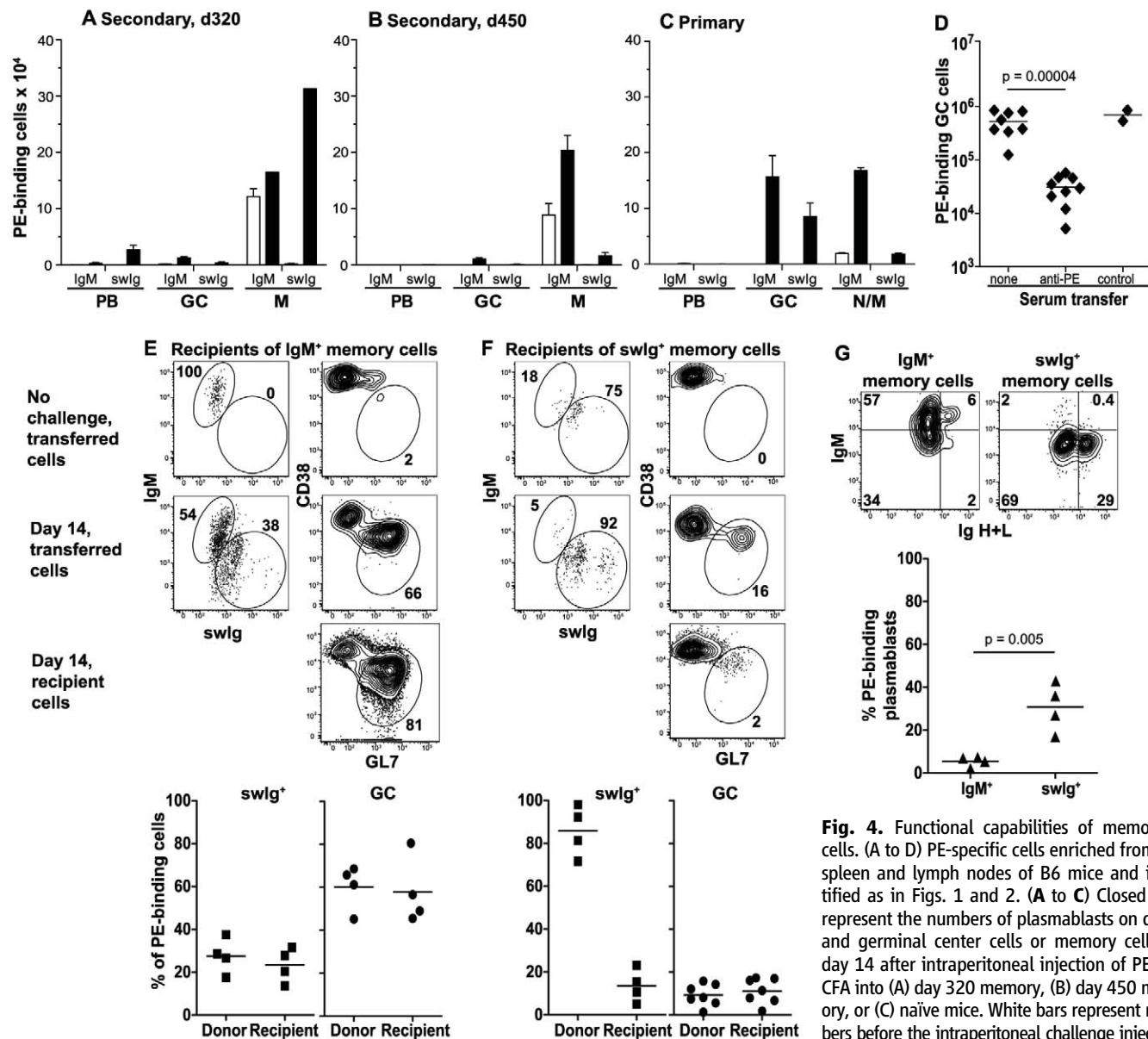


Fig. 4. Functional capabilities of memory B cells. (A to D) PE-specific cells enriched from the spleen and lymph nodes of B6 mice and identified as in Figs. 1 and 2. (A to C) Closed bars represent the numbers of plasmablasts on day 4 and germinal center cells or memory cells on day 14 after intraperitoneal injection of PE and CFA into (A) day 320 memory, (B) day 450 memory, or (C) naïve mice. White bars represent numbers before the intraperitoneal challenge injection. The mean and SEM ($n = 3$ to 4) are shown for all

groups except for the day 320 memory cells after challenge, where $n = 2$, and the mean is shown without error bars (range = 102,678). (D) Numbers of PE-binding germinal centers present on day 12 to 14 in mice left untreated or injected with serum from mice immunized with PE or control antigens (chicken ovalbumin or allophycocyanin) on days 3 to 6 after antigen injection. Statistical significance was established with an unpaired Student's t test. (E and F) Flow cytometric analysis of PE-specific cells of donor or recipient origin in the spleen and lymph nodes of CD45.1⁺ recipients of IgM⁺ (E) or swIg⁺ (F) CD45.2⁺ memory cells before and 10 to 14 days after PE and CFA challenge. Gates used to identify IgM⁺, swIg⁺, and germinal center cells are shown. Scatter plots show the percentage of the indicated cell types present in the PE-specific populations of donor or recipient origin in individual mice 10 to 14 days after PE and CFA challenge. (G) PE-specific cells derived from transferred IgM⁺ or swIg⁺ memory cells in individual recipient mice 5 days after PE and CFA challenge. Plasmablasts were identified as Ig^{high} cells. Statistical significance was established with an unpaired Student's t test (10).

long as swIg⁺ memory cells were present at the time of challenge. Therefore, the secondary response was tested in mice that were primed with PE 450 days earlier and contained 100,000 PE-specific IgM⁺ and scarcely any swIg⁺ memory cells. These mice generated very few swIg⁺ cells of any kind after challenge, which indicated that the IgM⁺ memory cells did not undergo isotype switching. The IgM⁺ memory cells increased only twofold after challenge (Fig. 4B), in contrast to the robust primary response of naïve IgM⁺

cells to intraperitoneal injection of PE, which generated many IgM⁺ and swIg⁺ germinal center and memory cells (Fig. 4C).

Several lines of evidence suggested that the poor secondary response of IgM⁺ memory cells was related to anti-PE immunoglobulin present during challenge. Injection of hyperimmune serum containing anti-PE immunoglobulin inhibited the generation of germinal center cells from naïve cells (Fig. 4D). In addition, purified IgM⁺ memory cells underwent isotype switching and

germinal center cell formation when transferred into naïve recipient mice and then challenged with PE (Fig. 4E). In contrast, purified swIg⁺ memory cells responded to PE in naïve recipient mice, as they did in immune mice, by producing memory cells but very few germinal center cells (Fig. 4F). Notably, the presence of swIg⁺ but not IgM⁺ memory cells inhibited germinal center formation by the naïve cells of the adoptive recipients (Fig. 4, E and F). This inhibitory effect correlated with rapid production

of plasmablasts by swIg⁺ but not IgM⁺ (Fig. 4G) memory cells after challenge. Thus, IgM⁺ memory cells were not intrinsically hyporesponsive in immune hosts but were functionally inhibited by antigen-specific immunoglobulins produced either before challenge by plasma cells or after challenge by memory cell-derived plasmablasts. Inhibitory FcγRIIb (23) was probably not involved, because IgM⁺ and swIg⁺ memory cells expressed equal amounts of this receptor (fig. S2A).

PE- and allophycocyanin-specific naïve B cells accounted for about 1:5000 and 1:25,000 of all B cells in mice. These high frequencies are likely related to the presence of multiple epitopes on these large multimeric proteins (24). It will be of interest to use the enrichment approach to enumerate naïve B cells specific for monomeric antigens, although antigen multimerization may be required (25).

Naïve PE-specific B cells generated IgM⁺ memory cells after immunization with PE and the adjuvants CFA (Fig. 2K), lipopolysaccharide, or alum (fig. S4), which suggested that this is a general feature of the primary immune response. These memory cells had few mutations in their IgM molecules, which indicated inefficient selection in germinal centers. It is possible that these poorly mutated IgM molecules had a high enough natural affinity for PE to trigger memory cell differentiation before extensive somatic mutation could occur.

The remarkable stability of the IgM⁺ memory cells compared with swIg⁺ memory cells was not related to selective enrichment of IgM⁺ cells (fig. S5A), migration of swIg⁺ cells to bone marrow (fig. S5B), or homeostatic proliferation (fig. S5C). The instability of swIg⁺ memory cells may be

related to inhibitory signals through TACI (fig. S2A) (21) or deleterious off-target mutations induced by AID (26). Despite being shorter-lived and outnumbered by IgM⁺ memory cells, swIg⁺ memory cells dominated the secondary response because of a capacity to be activated in the presence of high-affinity neutralizing serum immunoglobulin. However, even swIg⁺ memory cells could not produce germinal center cells, perhaps because their plasmablast progeny secreted enough immunoglobulin to clear the antigen very quickly. The failure to be activated efficiently in the face of immunoglobulin from swIg⁺ memory cells or plasma cells suggests that IgM⁺ memory cells do not contribute to the secondary response until these molecules decline. Serum immunoglobulin induced by certain subunit vaccines has been reported to decrease over time in humans (27), which suggests that IgM⁺ cells could become the reservoirs of humoral immune memory for these vaccines. Because of their lower affinity and ability to produce germinal center cells, IgM⁺ memory cells may also be useful for responding to antigenic variants produced by mutating pathogens.

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Supporting Online Material

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Enhancement of Consolidated Long-Term Memory by Overexpression of Protein Kinase Mζ in the Neocortex

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Todd Charlton Sacktor,² Yadin Dudai^{1*}

Memories are more easily disrupted than improved. Many agents can impair memories during encoding and consolidation. In contrast, the armamentarium of potential memory enhancers is so far rather modest. Moreover, the effect of the latter appears to be limited to enhancing new memories during encoding and the initial period of cellular consolidation, which can last from a few minutes to hours after learning. Here, we report that overexpression in the rat neocortex of the protein kinase C isozyme protein kinase Mζ (PKMζ) enhances long-term memory, whereas a dominant negative PKMζ disrupts memory, even long after memory has been formed.

Amnesic agents impair fresh memories during encoding and consolidation, and some can block reactivated long-term memories at reconsolidation (1). Furthermore, se-

lective brain lesions result in extensive loss of remote memories (2). Some agents have been proposed as potential memory enhancers, but their beneficial effect seems to be limited to the

encoding and immediate consolidation period (3–7). The importance of memory enhancement for treating cognitive decline calls for an intensive exploration of the effect of manipulating components of the memory storage machinery on memory performance.

Protein kinase Mζ (PKMζ) is a persistently active, atypical protein kinase C isoform that is critical for maintaining the storage of long-term memory long after its initial consolidation (8, 9). We have previously reported that inhibition of PKMζ in the insular cortex (IC) of the behaving rat by the pseudosubstrate zeta inhibitory peptide (ZIP) leads to erasure of long-term memory of conditioned taste aversion (CTA), an associative type of memory, up to at least 3 months after encoding, without affecting the ability of the rat to relearn the same association and without

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impairing recognition memory (10, 11). The training itself leads to a persistent increase in the level of the endogenous PKM ζ protein in the IC (fig. S1). We wanted to investigate the effect on CTA memory of targeted modulation of the level of PKM ζ in the IC. We therefore designed and generated lentiviruses expressing PKM ζ (LV_{PKM ζ}) or a dominant negative (DN) version of PKM ζ mutated at the active domain (LV_{DN}, PKM ζ -K281W) (Fig. 1A), and microinfused them, or control lentiviruses (LV_{GFP}) (GFP, green fluorescent protein), bilaterally into the IC (Fig. 1, B

and C). Under the conditions used, $43 \pm 9\%$ of the neurons (identified by NeuN staining, $n = 6$ hemispheres) at the core of the injected volume expressed the GFP reporter. Overexpression of the PKM ζ protein was evident (Fig. 1D).

Rats infected in the IC with LV_{DN} 6 days after single-trial CTA training and tested for CTA memory 7 days later displayed a marked reduction in memory (Fig. 2A). In parallel in vitro experiments, the DN inhibited PKM ζ activity tested on an exogenous kinase substrate (fig. S2). These data support our previous conclusion that

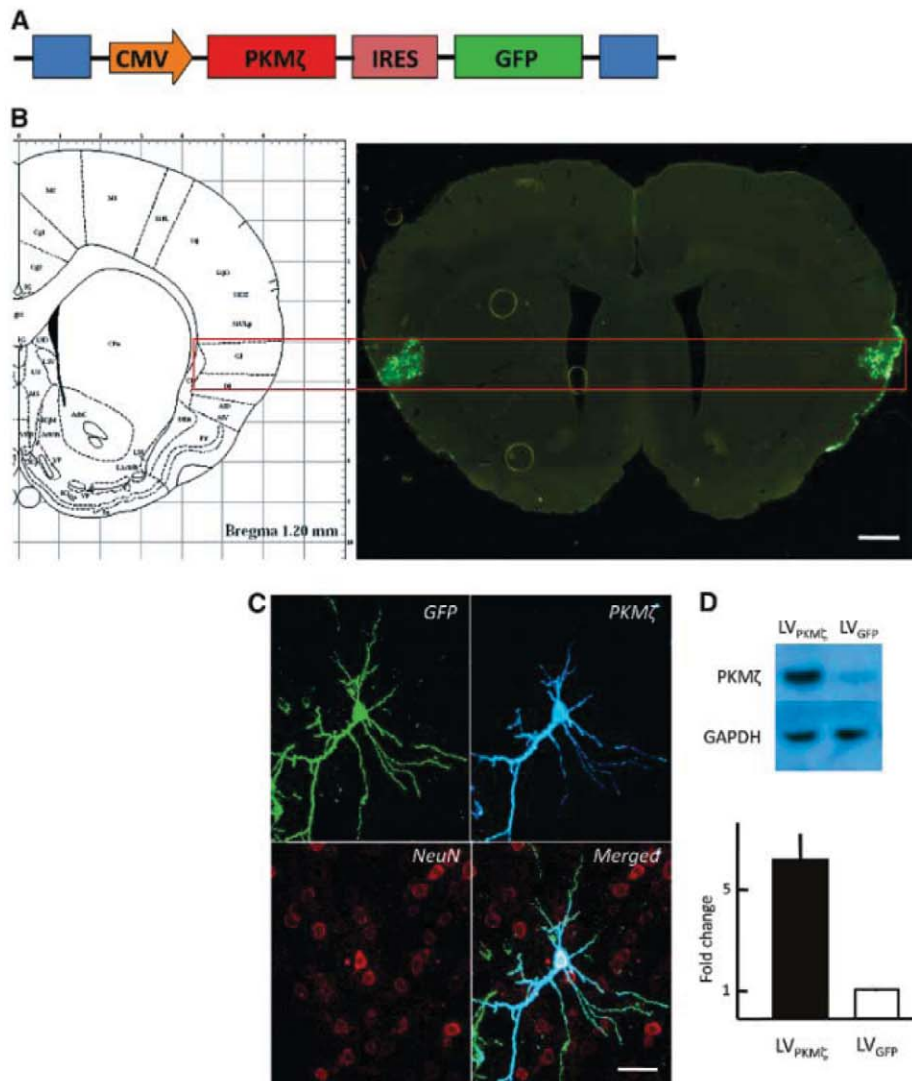


Fig. 1. Overexpression of PKM ζ in the IC. (A) Schematic map of the lentiviral construct designed to overexpress PKM ζ and GFP. The PKM ζ gene (or the DN mutation, PKM ζ -K281W) is under the control of a cytomegalovirus (CMV) promoter, followed by an internal ribosome entry site (IRES) and a GFP reporter. (B) A representative picture of a coronal section of the rat brain (bregma = 1.2 mm, 25 μ m width section), depicting GFP-infected cells in the bilateral IC. Scale bar, 100 μ m. (C) A neuron stained with GFP (green), PKM ζ (blue), and NeuN (red) and the merged picture of all the above. To allow clear single-neuron presentation, the picture was taken toward the periphery of the infected area. Scale bar, 20 μ m. (D) Upper panel: Western blot with an antibody specific for the catalytic domain of PKM ζ , depicting PKM ζ expression in the IC infused with the lentiviral vector containing the PKM ζ gene (LV_{PKM ζ}), compared to that infused with GFP alone (LV_{GFP}). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Lower panel: Quantification of the increase in PKM ζ expression as a consequence of infection with LV_{PKM ζ} .

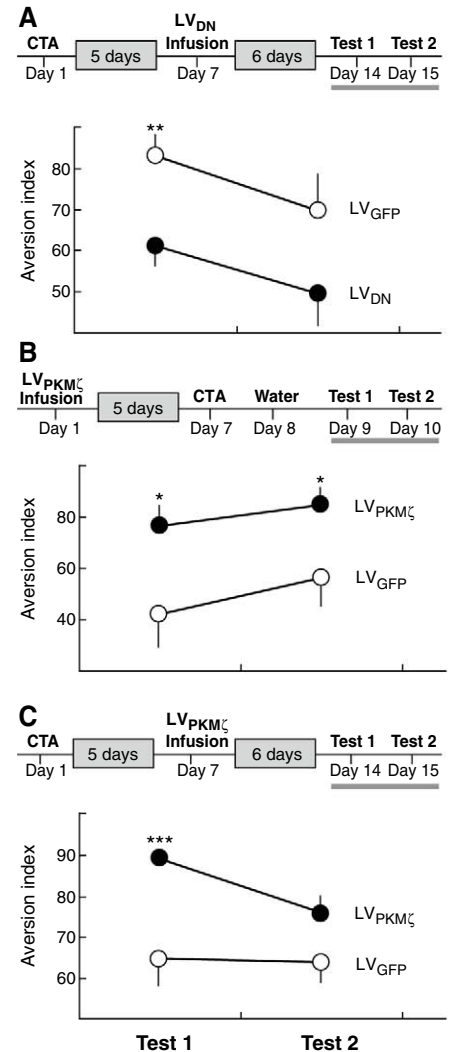


Fig. 2. Expression of a DN mutation of PKM ζ blocks, whereas overexpression of PKM ζ enhances, long-term memory in the IC of the behaving rat. (A) The IC of rats was infused bilaterally with LV_{DN} (Fig. 1) 6 days after CTA training, followed by testing 7 days later (two subsequent tests, a day apart, were used to quantify experimental extinction). Memory was significantly disrupted by DN PKM ζ overexpression. Here and below, test days are underlined in the protocol flowchart. LV_{GFP}, $n = 9$; LV_{DN}, $n = 10$. (B) LV_{PKM ζ} was infused bilaterally into the IC 6 days before CTA training, using a weak US to circumvent the ceiling effect of the conventional CTA training and hence permit detection of potential memory enhancement (see methods in the SOM). Memory was tested starting 2 days after training. Memory was significantly enhanced, and no extinction was evident. LV_{GFP}, $n = 7$; LV_{PKM ζ} , $n = 9$. (C) LV_{PKM ζ} was infused bilaterally into the IC 6 days after CTA training, and memory was tested starting a week later. Memory was significantly enhanced by overexpression of PKM ζ , and extinction is evident. LV_{GFP}, $n = 23$; LV_{PKM ζ} , $n = 28$. * $P < 0.05$, *** $P < 0.01$, **** $P < 0.001$. In (C), the statistics are for experimental versus control on test 1 and experimental on test 1 versus test 2.

the effect of ZIP on CTA memory in the IC is due to the inhibition of PKM ζ (10, 11).

Whereas infection of the IC with LV_{DN} disrupted long-term CTA memory, overexpression as a consequence of infection with LV_{PKM ζ} significantly enhanced long-term memory. Memory enhancement was positively correlated with the extent of LV_{PKM ζ} infection in the IC (fig. S3). It was evident both when the infection was performed before CTA training (Fig. 2B) and when performed at day 7 after CTA training (Fig. 2C), a time point when the long-term CTA trace in the IC is already consolidated (12). Because the strong unconditioned stimulus (US, LiCl) used in conventional CTA training results in a ceiling effect that could mask potential memory enhancement, in the LV_{PKM ζ} experiments we used a diluted LiCl solution as the US (13). This US produced little CTA memory when tested at day 7 after training [aversion index (AI) = 64.1 ± 4.7 , $n = 7$ rats], which is significantly lower than that observed in the PKM ζ -overexpressing group tested a week later (Fig. 2C; AI = 89.1 ± 2.5 ,

$P < 0.001$). Hence overexpression of PKM ζ did not retard the normal fading of memory but rather enhanced the already faded memory. To exclude the possibility that the overexpression shifted the cortex into a persistently aversive state, in which continual expression of the aversion would mimic stronger CTA memory, we tested the effect of microinfusion of LV_{PKM ζ} into the IC on aversion to a saccharin conditioned stimulus (CS) in the absence of CTA training. No significant effect on aversion was detected when infection was performed in the IC of naïve rats before they had ever encountered saccharin (Fig. 3A) or in the IC of rats that had previously been exposed to saccharin (Fig. 3B). We also did not detect differences in the volume of liquid consumed in the instrumental situation of the CTA conditioning protocol by the rats infected in the IC with LV_{PKM ζ} (total liquid consumed on the first exposure to saccharin, LV_{PKM ζ} = 8.29 ± 0.58 ml, LV_{GFP} = 8.36 ± 0.69 ml, $n = 18$ and 15 , respectively). We thus conclude that overexpression of PKM ζ in the IC has no significant effect on the sensorimotor or motivational faculties required to express CTA.

Does PKM ζ overexpression affect only the most recently acquired memory? The answer is no. When trained consecutively on two CSs with clearly distinguishable taste qualities, saccharin and NaCl (11), overexpression enhanced the long-term memory of both (Fig. 4).

Our data show that whereas inhibition of PKM ζ in the IC by competition with a DN mutation of the enzyme disrupts long-term CTA memory, overexpression of PKM ζ in the IC enhances memory, including memory formed long before the enzyme was overexpressed. Recently, a model was proposed to account for the erasure of memory by the inhibition of PKM ζ , according to which disruption of persistent activity of the enzyme disrupts trafficking of GluA2-containing AMPA receptors into the postsynaptic membrane and hence annuls the use-dependent increase in synaptic efficacy (9, 14). The inhibition of memory by the DN thus makes sense. But how does overexpression of PKM ζ increase memory already formed?

Considering the functional systems level; that is, what it is that the brain now does differently, three types of possibilities come to mind. One is that overexpression of PKM ζ enhances global, item-invariant operations, such as attention, incentive valence, or sensory or motor operations, all of which are required for memory expression but are not memory per se. Nevertheless, as noted above, there was no significant effect of infection with LV_{PKM ζ} in the IC on sensorimotor and motivational attributes of either naïve or CS-exposed rats, as manifested in their normal approach to and consumption of saccharin and in their total liquid consumption. Rats whose IC was microinfused with LV_{PKM ζ} 6 days before training, as in Fig. 2B, displayed a normal immediate reaction to LiCl, augmenting the conclusion that the treatment and overexpression of PKM ζ did not significantly alter sensorimotor, including visceral, responses relevant to CTA [supporting online material (SOM)]. Furthermore, in rats whose IC was infected with LV_{PKM ζ} on day 7 after conditioning, the enhanced long-term memory was still capable of experimental extinction, similar to a normal CTA trace obtained after the conventional, one-trial training (15) (Fig. 2C), implying no persistent shift in the reaction to the CS. All in all, the aforementioned data favor an effect on mnemonic functions. We cannot, however, completely exclude the possibility that overexpression of PKM ζ culminates in alterations of fine-tuning or implicit properties of the system that might amplify memory performance, yet are undetected in the nonmnemonic tests. A second possibility is that overexpression of PKM ζ results in the enhancement of global, item-invariant memory operations, of the type postulated to take part in memory retrieval, such as retrieval and search mode (16, 17). Finally, a third possibility is that overexpression of PKM ζ results in strengthening of item-variant memory operations (17), such as specific associations of specific items. The encoding of CTA in the IC was reported to be distributed, and specific associations are estimated to engage plastic changes in about 25% of the neurons (18), suggesting that the level

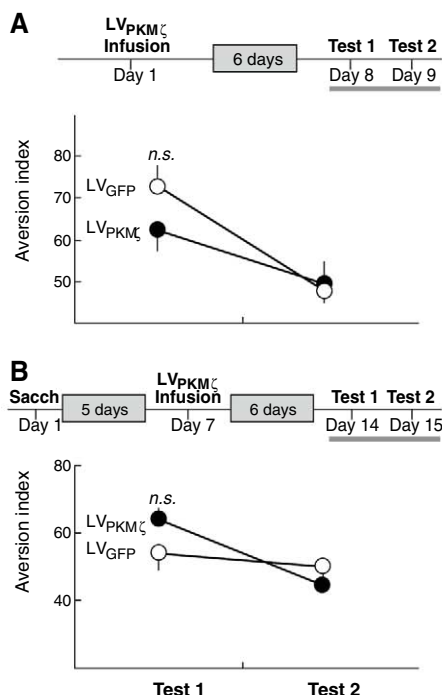


Fig. 3. Overexpression of PKM ζ in the IC does not alter innate preferences for saccharin. **(A)** The IC of rats was microinfused bilaterally with LV_{PKM ζ} or LV_{GFP}, followed by a multiple choice test (see methods in the SOM) for the preference for saccharin versus water on days 8 and 9. There was no significant difference between the groups in their preference for saccharin (see methods in the SOM). LV_{GFP}, $n = 16$; LV_{PKM ζ} , $n = 17$. **(B)** Rats were first exposed to saccharin, followed 6 days later by infusions into the IC of either LV_{PKM ζ} or LV_{GFP}. Preference for saccharin was tested 7 and 8 days later. Again, there was no significant difference between the groups in their preference for saccharin. LV_{GFP}, $n = 25$; LV_{PKM ζ} , $n = 26$.

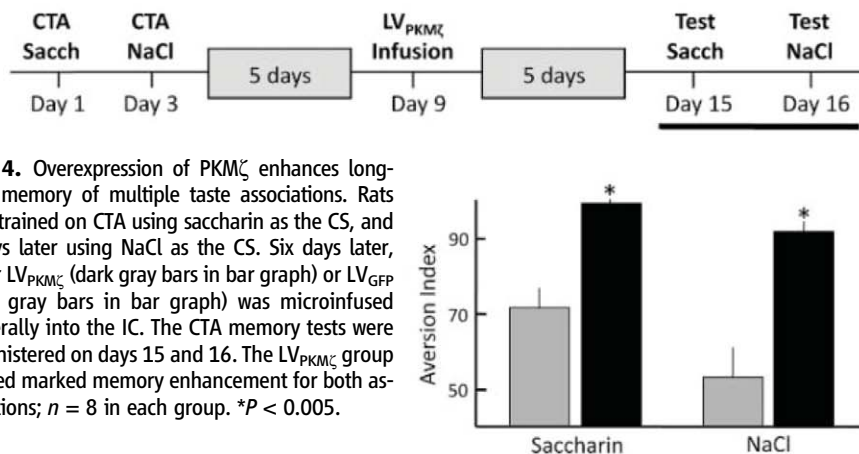


Fig. 4. Overexpression of PKM ζ enhances long-term memory of multiple taste associations. Rats were trained on CTA using saccharin as the CS, and 2 days later using NaCl as the CS. Six days later, either LV_{PKM ζ} (dark gray bars in bar graph) or LV_{GFP} (light gray bars in bar graph) was microinfused bilaterally into the IC. The CTA memory tests were administered on days 15 and 16. The LV_{PKM ζ} group showed marked memory enhancement for both associations; $n = 8$ in each group. * $P < 0.005$.

of overexpression that we obtained in the IC in the present study could affect multiple behaviorally relevant representations. The possibility that we indeed achieved strengthening of item-variant memory operations is perhaps the most exciting, but at this point in time we cannot dissociate item-invariant from item-variant effects, particularly given that overexpression appears to enhance more than one stored association.

As to the hardware implementation level of consolidated memory enhancement by PKM ζ , it is implausible that this is due to flooding the cortex indiscriminately with the overexpressed enzyme, because this could lead to nonselective change in synaptic weights or even to saturation of use-dependent plasticity. Indeed, saturating amounts of exogenously applied PKM ζ , when perfused directly into neurons, appear to produce potentiation of all of the cell's synapses (19). However, PKM ζ synthesized in neurons is captured at recently active synapses, by a process of synaptic tagging, enabling these specific synapses to maintain a persistent state of enhanced efficacy (20). Thus, it is plausible to assume that the overexpressed kinase may augment the endogenous increase (fig. S1). We did observe differential accumulation of overexpressed PKM ζ in dendritic spines in the IC in vivo (fig. S4), which is consistent with but does not prove the hypothesis. Selectivity resulting in strengthening memory might hence be achieved if the new enzyme molecules synthesized by the overexpressed gene are preferentially attracted to tagged synapses.

The enhancement of memory long after encoding also raises the possibility that synaptic tagging and capture (21, 22) can mold memory over much longer periods of time than previously supposed, a mechanism that might be useful in situations such as the integration of new episodic items into long-term memory schemata (23) or, more generally, memory summation over prolonged periods of time (24).

The observation that overexpression of PKM ζ enhances memories long after they had been formed renders it plausible to consider PKM ζ a potential target not only for memory blockers (which might be useful, for example, in treating post-traumatic stress) but also for novel types of memory enhancers in the treatment of amnesia and cognitive decline.

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Supporting Online Material

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Materials and Methods

SOM Text

Figs. S1 to S4

References

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Forensics Follows New Clues

Forensic DNA analysis has already revolutionized criminal investigation. Now researchers and toolmakers are building faster, more sensitive DNA fingerprinting platforms while adding entirely new techniques to detectives' toolkits.

By Alan Dove

On television, all forensic technology fits into a standard plotline. Someone commits a brilliant, dastardly crime, and a team of romantically entwined investigators—with limitless access to cutting-edge tools—finds and collars the perpetrator in under an hour, with time for commercial breaks.

Reality, of course, is considerably messier. The majority of criminals are not especially brilliant, but even their rudimentary efforts to cover their tracks can make an investigator's job infuriatingly difficult. New technology might help, but only if crime labs can adopt it without breaking their budgets or having to retrain an entire staff.

The problems are as diverse as the cases. DNA analysis forms the backbone of modern forensics, but labs processing DNA evidence operate under enormous technical and legal constraints. The slightest contamination of a sample, or just poor recordkeeping, can invalidate the evidence from multiple cases. Meanwhile, technicians are often working against the clock; if a prime suspect is in custody, there may be only a few hours to process evidence before he or she is released on bail.

DNA fingerprinting isn't the only challenge, though. Out in the field, investigators spend a lot of time searching for bodies, and then trying to identify them even if they're badly decomposed.

To address these problems, researchers working in corporate, university, and government labs are modifying established forensic technologies and are also developing entirely new ones. While some of the tools coming from this interdisciplinary field are targeted specifically at solving crimes, others may find a broad range of uses in basic and applied research.

CSI IN A CARTRIDGE

For researchers at **Life Technologies** in Carlsbad, California, developing new forensic technologies is a longstanding business interest. The company offers a range of DNA analysis products and reagents, including the popular PrepFiler DNA extraction system. "The PrepFiler kit chemistry is a magnetic bead based extraction system, but the magnetic particles are optimized, they have a multicomponent surface chemistry that really maximizes the binding of DNA to the beads in order to maximize recovery," says Lisa Calandro, senior staff scientist in the Human Identification and Forensics Group at Life Technologies.

The pre-made kit simplifies the DNA isolation somewhat, but the protocol still requires multiple steps, each of which introduces the opportunity for errors, spills, and contamination.



There is little doubt that researchers in the field will continue to push the technology in new directions.

tion. To reduce those risks, Calandro and her colleagues have now developed an entirely automated version called AutoMate ExpressT.

"What the AutoMate ExpressT does is it takes that chemistry and puts it into a cartridge-based system, and then the extraction procedure is performed on a [benchtop] instrument," Calandro explains. Besides eliminating operator error from the protocol, the new system also makes it faster and simpler. A technician can simply cut the tip from a cheek swab, lyse the cells on it, centrifuge them through a separation column, and then apply the solution to the AutoMate ExpressT column. Completely purified DNA emerges from the other end.

While simple purification works well if the DNA came directly from a suspect, evidence from a crime scene requires special handling. Besides having much lower concentrations of DNA, crime scene samples may include mixtures of suspect and victim cells, as well as environmental chemicals that can inhibit polymerase chain reactions (PCR). **continued »**

UPCOMING FEATURES

Synthetic Genomics—March 25

Small Devices—April 1

Cell Separation Technologies—May 6

FEATURED PARTICIPANTS

Federal Bureau of Investigation
www.fbi.gov

Forensic Science Service
www.forensic.gov.uk

Karolinska Institute
www.ki.se

Lawrence Livermore National Laboratory
www.llnl.gov

Life Technologies
www.lifetechnologies.com

National Institute of Standards and Technology
www.nist.gov

PerkinElmer
www.perkinelmer.com

Promega
www.promega.com

U.S. Department of Homeland Security
www.dhs.gov

University of Arizona
www.arizona.edu



POLICE LINE DO NOT CROSS

After purifying DNA from these samples, technicians might turn to a system such as the Life Technologies Quantifiler Duo, which quantifies the ratio of male to female DNA in a sample as well as detecting any PCR inhibitors. "This can be very helpful in sexual assault cases, where you have potentially very low amounts of male DNA," says Calandro.

Both the AutoMate ExpressT and Quantifiler systems are already in use by many forensics laboratories, but the company is also working on additional tools to speed the forensic DNA workflow. Though DNA evidence was previously considered a tool for solving homicide and assault cases, Calandro says police departments are increasingly using DNA fingerprints in property crimes such as burglary. That increases pressure on labs to boost their sample processing capacity, without compromising reliability.

The standards for DNA fingerprinting are also changing. Since 1994, when the U.K.'s **Forensic Science Service (FSS)** pioneered DNA fingerprinting (the FSS' closure was recently announced: <http://scim.ag/g19eJX>), European law enforcement officials have steadily added more genetic loci to the continent's standard testing protocols, with some variation between countries. As a result, European samples may be tested for matches at up to 16 distinct loci. Meanwhile, the **U.S. Federal Bureau of Investigation (FBI)** has settled on a standard protocol with 13 loci that partially overlap with the European set, but FBI officials are considering adding more.

Fortunately, forensics toolmakers are already addressing that challenge. **Promega** in Madison, Wisconsin for example, now offers the PowerPlex 16 system, a comprehensive DNA analysis platform that analyzes the 13 FBI loci plus three additional ones, overlapping with European testing standards and also providing gender identification. The company markets the system for a wide range of applications, from forensics and paternity testing to cell culture identification. Besides screening more loci than conventional kits, the PowerPlex 16 provides greater sensitivity and speed than traditional DNA fingerprinting protocols.

BOOK 'EM, DNA

Even with streamlined sample preparation, performing a complete DNA fingerprinting analysis can take a long time. "The overall process, in the way that is typically used in the industry, can take up to 14 days," explains Frederic Zenhausern, Ph.D., director of the Center for Applied NanoBioscience and Medicine at the **University of Arizona** in Phoenix.

Zenhausern and his colleagues in collaboration with the U.K. FSS have now developed a microfluidic device that can shorten the steps between purifying DNA and reading a fingerprint to two hours, while also making it more straightforward for technicians. "Everything is piloted by the computer, and it's a very simple user interface," says Zenhausern.

At the heart of the device is a microfluidic chip built from previously tested technologies. "What we do here is combine inside a lot of different components that have been developed in the past," says Zenhausern. Thermally activated polymers act as valves to move the sample from one step to the next, and on-board sensors allow the attached computer to control everything from reagent mixing to PCR and electrophoresis.

The chip is part of an enclosed, single-use cartridge. "All the pumping and valving systems are self-contained within the cartridge ... that was one of the key elements the forensic community really wanted, to really be sure there is no potential for cross-contamination," says Zenhausern. He adds that while the fluid volumes are small compared to traditional laboratory techniques, the team deliberately avoided pushing the boundaries of miniaturization; for forensics labs, reliability is more important than portability.

Indeed, confirming that the system is reliable enough to meet the rigorous standards for criminal evidence processing is the next major hurdle. "The Forensic Science Service in the United Kingdom has been really leading the development for us, they really have focused on the [reliability] measurements with the concept of where those measurements will go in the legal system," Zenhausern says.

Besides validating the new technique, the investigators are collaborating with a laboratory equipment vendor to mass-produce the microfluidic cartridges and their associated hardware. Ultimately, Zenhausern hopes to get the price per test down to twenty dollars or less, with a system that could be used in a police station's booking room rather than a crime lab. At that point, DNA testing could become as routine as a mug shot, allowing police to confirm or exclude a suspect before the booking process is even finished.

The system could also find uses outside of criminal justice. "If you change the design of the cartridge and the assay chemistry, the same instrumentation platform can be used for other applications in other market segments, like clinical diagnostics," says Zenhausern.

When the new microfluidic system reaches the market, it will already have competition. At **PerkinElmer** in Waltham, Massachusetts, product developers have been working hard to streamline forensic DNA analysis, resulting in the company's innovative JANUS workstations. The systems use PerkinElmer's proven liquid-handling automation platform to handle every step of DNA analysis, from purification through quantitative PCR setup. Because the system is modular, labs can acquire it piecemeal, and potentially add other processes in the future.

PAGING MR. HOFFA

While DNA evidence is useful for identifying perpetrators, in many cases police also have to grapple with the problem of

identifying—or even finding—victims. Bodies buried in hidden graves are particularly challenging; once the soil has settled, the gravesite may be impossible to see. Ground-penetrating radar, search dogs, and soil sampling can help, but each technique comes with severe limitations. None of these methods can search large areas efficiently, and bodies buried underneath concrete slabs elude all of them.

Using a clever high-efficiency sampling technique, researchers at the **National Institute of Standards and Technology** (NIST) in Boulder, Colorado, may have found a better approach. The method builds on a standard air-sampling strategy used in a variety of environmental sensing experiments. “A lot of people use purge and trap, whereby they pull a sample into an empty tube and trap with liquid nitrogen anything that might be volatile. What we do is instead of using a one-sixteenth-inch tube, we use a very small capillary tube, the inside periphery of which is actually coated with very strong absorbent,” explains Thomas Bruno, Ph.D., leader of Properties for Process Separations in the Thermophysics Division of NIST and principal investigator on the new work.

For grave soil detection, Bruno and his colleagues lined the capillary tube with aluminum oxide, which has a high affinity for the protein decomposition products that come from decaying flesh. A vortex tube connected to the system first chills the capillary to -40°C during sample collection then heats it to 160°C to elute the sample, which the device then quantifies in a sensitive colorimetric assay.

“If the compound is present at approximately 20 parts per billion in our substrate, we can detect enough or capture enough to get a solute concentration within 10 percent,” says Bruno, adding that “if all you’re interested in is a yes or no answer we can do it with two to three parts per billion, so it’s extremely sensitive.”

In the team’s initial proof-of-concept studies, the device could detect the bodies of decaying rats even 20 weeks after they had been buried. The investigators could even detect bodies underneath concrete slabs, simply by drilling a hair-thin hole through the slab and inserting the capillary tube. Besides the potential for solving modern-day crimes, the system might also help unearth historical graves. Bruno says he’s already been approached by a group hoping to recover the bodies of British explorer Robert F. Scott and two compatriots, who are believed to be entombed beneath an Antarctic glacier.

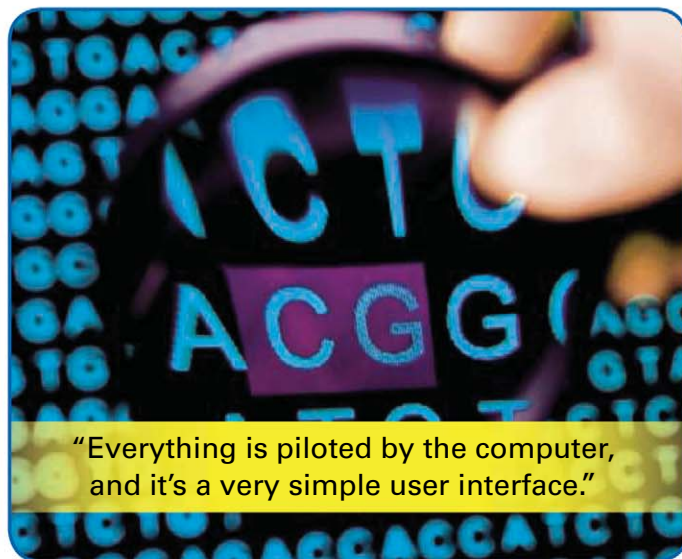
Because the key innovations lie in the sampling method, simple modifications could produce systems to sense a wide range of other chemicals. Indeed, the NIST researchers are already collaborating with the **U.S. Department of Homeland Security** on a portable, high-sensitivity explosive-detection system built on the same general design.

“We’ll probably have 60 or 100 capillaries in sort of a bundle, and the idea for Department of Homeland Security is: ‘can you use this to sample a larger volume of air that might be inside of a cargo container on board a ship?’” says Bruno.

TIMING TEETH FROM TRINITY

Applied research such as Bruno’s has produced many of the recent innovations in forensics, but basic science and serendipity have contributed as well. At the Lawrence Livermore National Laboratory in Livermore, California, for example, senior scientist Bruce Buchholz and his colleagues stumbled onto a new forensic tool while trying to answer a fundamental question in neuroscience.

“Maybe seven or eight years ago, I started working with



“Everything is piloted by the computer, and it’s a very simple user interface.”

Kirsty Spalding and Jonas Frisen at the **Karolinska Institute**, and we were looking at using the ^{14}C bomb pulse to date turn-over of neurons,” explains Buchholz. The “bomb pulse” was the global spike in atmospheric ^{14}C from aboveground nuclear testing at the height of the Cold War. People worldwide have taken the isotope into their tissues at predictable rates from the food supply, providing a potential clock for dating various cells’ life-spans.

Looking for a control tissue to compare with the neurons, Buchholz and his colleagues realized that the mineral component of tooth enamel does not turn over. As a result, the level of ^{14}C in the enamel reveals when it was formed; someone born in 1955 grew teeth with a much higher ^{14}C level than someone born in 1985. Because scientists have tracked the bomb pulse rigorously over the years, the team can analyze a single tooth and determine an individual’s birth date to the nearest year. Combining the method with an established technique called aspartic acid racemization could reveal both the approximate birth date and the approximate date of death of a cadaver.

Aboveground nuclear testing ended in 1963, so the technique will become less useful over time. Eventually, the isotope will dissipate and decay until ^{14}C levels return to their pre-pulse state.

Nonetheless, testing ^{14}C levels will likely remain useful for a while. “I suspect in 20 years it’s going to be so flat that it’s not going to be usable for something that’s contemporary,” says Buchholz, but he adds, “There’s still a lot of applications for people who’ve been alive over the course of the pulse.”

Whether future scientists’ foray into forensics are accidental, like Buchholz’s, or a deliberate career choice, like Callandro’s, there is little doubt that researchers in the field will continue to push the technology in new directions. As they do, real world crime scene investigators can look forward to more happy endings and fewer cliffhangers.

Alan Dove is a science writer and editor based in Massachusetts.

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Guangdong University of Technology is located in Guangzhou, a beautiful city in south China. It is a key multi-disciplinary university of Guangdong Province with a history of over 50 years. Taking the advantage of bordering on Hong Kong and Guangdong's prosperous economic development, the university's research ability and competitive power has been dramatically improved. To better implement the university's Talent Strategy, GDUT starts to set up the "One-Hundred Talents" Project which aims to recruit one hundred talents and 10 innovation teams from overseas within 3-5 years.

We are seeking candidates with a doctorate degree and meet one of the following conditions:

1. Experts or scholars serving as professors or at equivalent positions in foreign universities or research institutes, having experience in presiding over major technology projects; experts or scholars serving as associate professors or at equivalent positions who master key technologies or are urgently needed; young scholars having great potentialities.
2. Entrepreneurial talents having their own intellectual property rights or master core technologies, having overseas experience of setting up their own business, and are familiar with related areas and international rules; Professionals and technical talents holding senior positions in internationally-renowned companies; The university will work in association with the local enterprises to help fulfill both the talents' academic and marketable values by facilitating their innovation on campus and entrepreneurship in the "high-tech business incubator" based in the university.

The talents can serve in the university as full-time or part-time staff member, or as a team.

We offer competitive compensation and benefits to the selected candidates. The maximum annual income and the maximum R&D fee can amount to RMB1000,000 and RMB10,000,000 respectively. The laboratory space will be provided. We also provide the selected candidates a 100M2 temporary apartment, and for those who need to buy an apartment in Guangzhou, the maximum offered housing package can amount to RMB800,000.

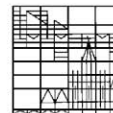
Interested and qualified individuals should submit the following documents:

- (1) a cover letter and a resume, including personal data, education background, work experience, professional title and achievement;
- (2) a statement of future plan for the post and the research interests;
- (3) a statement of personal requests to the University.

Contact information:

Contact person: Ms Zhiying Zeng, Telephone: 0086-20-39322792, Fax: 0086-20-39322208, E-mail: gduyzzp@gdut.edu.cn. Address: Human Resource Department, Guangdong University of Technology, No. 100 Waihuan Xi Road, Guangzhou Higher Education Mega Center, Panyu District, Guangzhou, P.R China. Postcode: 510006 Website: www.gdut.edu.cn

**UNIVERSITY
OF KONSTANZ**



The University of Konstanz is one of the nine Universities of Excellence in the Federal Republic of Germany.

The Department of Chemistry in the Faculty of Science invites applications and nominations for an outstanding candidate in the area of theoretical chemistry **starting October 1st, 2011** for a

**Professor of Theoretical Chemistry
(W3 - Tenured)**

For further information please visit our homepage:

<http://www.uni-konstanz.de/stellen>

Please send your application together with a curriculum vitae, a list of publications, and a statement of research and teaching interests under the reference number 2011/005 as one single *pdf-file* to:

Prof-2011-005@uni-konstanz.de at the latest by **March 31st, 2011**.

With a view towards increasing the number of female professors, the University of Konstanz specifically encourages qualified female candidates to apply.

Freie Universität Berlin



The Freie Universität Berlin (FUB) and the Leibniz-Institut für Molekulare Pharmakologie (FMP; Forschungsverbund Berlin e.V.) jointly invite applications for the following position:

**Full Professor (W3)
in Molecular Pharmacology (FUB)**
and

**Director of the Leibniz-Institut
für Molekulare Pharmakologie (FMP)**

We are seeking an outstanding scientist with high international visibility to lead the Leibniz-Institut für Molekulare Pharmakologie (www.fmp-berlin.de) into a new era. The FMP offers exceptional research opportunities including a screening unit for Chemical Biology and an NMR park connected to ESFRI projects (EU-OPENSOURCE, INSTRUCT), state-of-the-art animal facilities, mass spectrometry and laser-scanning microscopy. The candidate's research should be in an area with potential for drug target discovery, such as protein homeostasis, intracellular transport, transport across membranes, chromatin dynamics or other fields with high future pharmacological relevance. This research should complement and interlink with researchers at the FMP and FUB (www.fu-berlin.de) through joint research programs. The FMP is situated on the biomedical research campus in Berlin-Buch and offers a highly dynamic research environment covering all aspects of molecular pharmacology. It comprises groups working in the fields of ion channel and receptor physiology and pathology, chemical biology, structural biology, molecular imaging and biophysics. Researchers at the FMP enjoy unique opportunities for multidisciplinary work ranging from the atomic level to *in vivo* studies.

The position requires a doctoral degree, a distinguished record of research and teaching, and experience in leading an active research programme. The candidate must have demonstrated the ability to secure external funding, and show strong commitment to the mentoring of faculty, students and fellows. The successful candidate will be expected to teach 2 hours per week during term at the Institute of Pharmacy of FUB. The teaching language is either English or German and the working language at the FMP is English, allowing active communication among our internationally diverse faculty and colleagues.

FMP and FUB are equal opportunity employers and specifically welcome applications from female scientists. Preference will be given to applicants with disabilities when qualifications are equivalent. Applicants must fulfil the formal requirements of Berlin's university law (§ 100 Berliner Hochschulgesetz).

Please submit a full curriculum vitae, including a letter of motivation, statements addressing research and teaching interests and academic leadership goals, copies of five representative publications, and the names and contact information of three references, to Prof. Dr. H. H. Hilger, Dean, Department of Biology, Chemistry and Pharmacy, Freie Universität Berlin, Takustr. 3, 14195 Berlin, Germany (hard copy and email: dekan@bcp.fu-berlin.de). Applications will be considered until **April 15th, 2011**. For further inquiries please contact Prof. Dr. Hartmut Oeschkinat, acting director of FMP (oeschkinat@fmp-berlin.de).



**UNIVERSITY OF
LIVERPOOL**

**Faculty of Science and Engineering
Department of Chemistry**

**Postdoctoral Research
Associate (Photocatalysis)**

£30,747 - £35,646 pa

A two-year postdoctoral position is available in the development of new oxide nanoparticle visible light photocatalysts, in the research group of Professor M.J. Rosseinsky, FRS. A PhD in Chemistry or Materials Science and experience in photocatalyst measurement and nanoparticle synthesis is essential. X-ray diffraction and transmission electron microscopy in optical properties of materials is desirable.

Job Ref: R-574491/S

Closing Date: 1 April 2011

For full details, or to request an application pack, visit www.liv.ac.uk/working/job_vacancies/ or e-mail jobs@liv.ac.uk Tel 0151 794 2210 (24 hr answerphone) please quote job ref in all enquiries.

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We are looking for applicants with senior level experience who have a commitment to excellence and the energy, enthusiasm, and innovative thinking necessary to lead a dynamic and diverse organization.

The successful candidate for this position will be appointed at a salary commensurate with his/her qualifications. Full Federal benefits will be provided including leave, health and life insurance, long-term care insurance, retirement, and savings plan (401k equivalent).

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If you are ready for an exciting leadership opportunity, please see the detailed vacancy announcement at <http://www.jobs.nih.gov> (under Executive Jobs).



Applications must be received by April 29, 2011.



Boston University School of Medicine

The Whitaker Cardiovascular Institute
of Boston University Medical School,
www.bumc.bu.edu/busm-wci,
is seeking applicants for its

NIH Funded "Multidisciplinary Training in Cardiovascular Research" Program

Applicants should have an MD or PhD and
should be seeking a training position with a
cardiovascular interest for at least two years.

**Contact Nancy Clinton,
ncClinton@bu.edu for an application.
Due to federal regulations, all
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card status at time of application.**

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www.bumc.bu.edu



Ernst Strüngmann Institute
in Cooperation with Max Planck Society



The Ernst Strüngmann Institute (ESI) and the Max Planck Society announce an

ESI / Max Planck Research Group

in the area of "Optogenetics in Cognitive and Systems Neuroscience"

The ESI conducts brain research with the aim to understand how the many parts of the nervous system work together to bring about brain function and behavior. The activity of individual neurons or brain areas has been documented in great detail. Yet, we are only beginning to understand how neuronal activations are brought about by interactions among neurons and brain areas, and how changes in those interactions subserve adaptive behavior. Optogenetic techniques hold the potential to shine light onto those processes, through the simultaneous assessment of activity in large numbers of neurons and/or the excitation or inhibition of defined neuronal groups. The potential cell type specificity of optogenetic stimulation and recording methods adds an important layer of resolution to systems and cognitive neuroscience.

The Research group will focus on the application of optogenetic methods to address central questions in cognitive and systems neuroscience. It will use in-vivo approaches in a mammalian model system. Candidates with expertise and/or interest in using the non-human primate model are particularly encouraged to apply. The Research Group will be established at the ESI (www.esi-frankfurt.de), a brain research institute with the format of a Max Planck Institute, which is financed by the Ernst Strüngmann Foundation and operating in close cooperation with the Max-Planck-Society.

Funding for the Research Group covers a set-up package, the position of the group leader, a technician, one PhD and one post-doctoral fellowship, secretarial support, plus consumables, and is (initially) for 5 years. Candidates should have a successful track record including in-vivo optogenetics. Applications should include a CV, a list of publications, a one-page summary of scientific achievements, a two-page research plan and two letters of recommendation.

Successful candidates will be invited to a symposium on 10 May 2011 at the ESI in Frankfurt.

The ESI provides a high-performance data storage and compute facility, an animal facility that includes a non-human primate facility, and access to the nearby Brain Imaging Center (BIC) with two research dedicated MRI and one MEG system. The Max Planck Society for the Advancement of Science is an independent, non-profit research organization that primarily promotes and supports basic research. The society currently operates 80 institutes and research facilities with more than 23,400 employees, including 4,400 scientists. The ESI and the Max-Planck-Society are committed to equal opportunities and to employing disabled persons.

Please send your application in PDF format no later than **April 8, 2011** to: office.fries@esi-frankfurt.de

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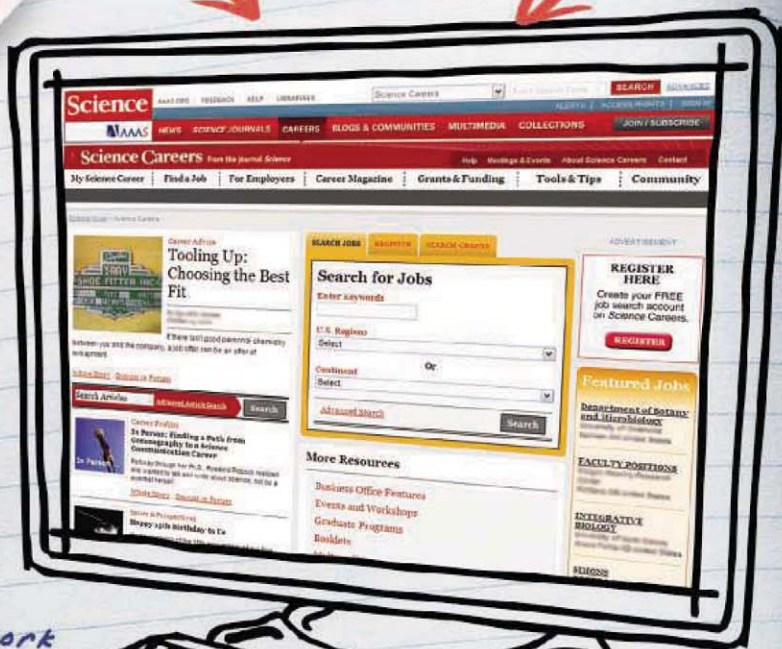
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- Fluid mechanics
- Solid mechanics and structures
- Thermal engineering
- Process (systems) engineering
- Electrical engineering
- Materials and nanotechnology
- Bioenergy/biomass



Focused preferably on energy and environment.

Our goal is to employ talent, building-up a multidisciplinary team of advanced engineering research located in Seville (Spain).

Requirements:

- PhD Engineers.
- Highest level of research capacity.
- Significant international experience in prestigious entities in this field (preferably USA).
- Recent publication and/or patent activity.
- Minimum experience of 7 years in R&D.
- English fluency.
- Academic and company R&D experience are an advantage.

To apply for this position, please send resume to abengoaresearch@abengoa.com



ISTITUTO ITALIANO DI TECNOLOGIA
ISI GENOMICS CENTER OF GENOMIC SCIENCE

Istituto Italiano di Tecnologia Outstation on Genomic Science at the European School of Molecular Medicine (IIT@SEMM), IFOM-IEO Campus, Milan, Italy

The Istituto Italiano di Tecnologia (IIT, <http://www.iit.it>) has recently created an IIT-Network comprising nine outstations at Italian research institutions. The **IIT@SEMM** outstation is affiliated with the European School of Molecular Medicine (SEMM; <http://www.semm.it>) at the **IFOM-IEO Campus** in Milan (<http://www.ifom-ieo-campus.it>), a vibrant research center dedicated to basic and translational cancer biology, home to state-of-the-art technological services. The focus of IIT@SEMM is on Genomic Science, and in particular Cancer Genomics (<http://genomics.iit.it>). The program includes a Genomic Unit, a high-throughput phenotype Screening Unit and a Computational Research Unit. Applications are invited for the following positions within the IIT@SEMM program. **Application deadline: March 28, 2011.**

In order to comply with the Italian law (art. 23 of Privacy Law of the Italian Legislative Decree n. 196/03), we kindly ask every candidate to provide his/her consent to allow IIT to process his/her personal data. The Istituto Italiano di Tecnologia is an Equal Opportunity Employer that actively seeks diversity in the workforce.

Group Leader, Computational and Systems Biology

The candidate will lead an independent research group, with full access to all the technological facilities of both IIT@SEMM and the IFOM-IEO Campus. Priority will be given to scientists developing **Computational and Systems Biology** approaches in Genomics, with or without wet-lab activity. Areas of interest include genome-wide mutational analysis, functional genomics, epigenome analysis, gene regulation (coding, non-coding or small RNAs; regulatory networks), or any other activity pertinent to Cancer Genomics. Applications will be considered at the Junior and Senior levels. According to IIT policy, this position will be subject to a Senior Researcher or Team Leader contract, depending on seniority.

Applications to this position should include the CV, names and e-mail addresses of three referees, and a personal statement focusing on future research plans (max. 3 pages), to be sent as a single Pdf file to Dr. Diego di Bernardo (dibernardo@tigem.it), chair of the Search Committee.

High-throughput sequencing Data Analyst, Computational Research Unit

This Unit is the core computing facility of IIT@SEMM, guaranteeing informatic development, implementation, training and support in Genomic research activities. Candidates must have previous experience with the analysis of high-throughput DNA sequencing data (e.g. ChIP-seq, RNA-seq), a strong background in statistics and familiarity with statistics packages such as R. The ideal candidate will have Programming skills, as well as a solid understanding in Molecular Biology, enabling him/her to productively interact with wet-lab biologists. Tasks will include providing support to scientists in annotating sequencing tracks with known genomic features; aiding the discovery of novel genomic features (e.g. exons, splice sites); transforming sequence tag counts into expression values; performing DNA motif searches, cluster analyses, meta-analyses, etc... This position will be subject to a Post-doctoral contract.

Applications to this position should include the CV, names and e-mail addresses of three referees, and a personal statement focusing on computational skills and interests (max. 2 pages), to be sent as a single Pdf file to Dr. Heiko Müller, coordinator of the Computational Research Unit (heiko.muller@iit.it).



**UNITED NATIONS
UNIVERSITY**

VACANCY ANNOUNCEMENT

**RECTOR, UNITED NATIONS UNIVERSITY
TOKYO, JAPAN**

About United Nations University (UNU): The UNU is the academic arm of the United Nations system. Its mission is to contribute through collaborative research and postgraduate education, dissemination of knowledge and advisory services, to efforts to resolve the pressing global problems of human survival, development and welfare that are the concern of the United Nations, its Peoples and Member States. The University functions as a think tank for the United Nations system and for UN Member States providing knowledge-based policy advice.

The Position: The UNU is searching for a new Rector. The Rector is the chief academic and administrative officer of the University and has overall responsibility for the direction, organization, administration and the programmes of the University. The position carries the rank of United Nations Under-Secretary-General (USG). The Rector is appointed by the Secretary-General of the United Nations after consultation with Director-General of UNESCO. The position requires extensive overseas travel. The continuing development and activities of the UNU places ever-increasing demands on the incumbent.

Required qualifications: Advanced university degree, preferably a Ph.D. in a discipline related to the human sciences, sciences of life, the earth and the biosphere. The successful candidate must have a prominent academic profile with evidence of high quality research work in the course of his/her career. Demonstrated management experience as the head of a university or research centre is required. Fluency in English and a working knowledge of French are essential.

Desirable qualifications and characteristics: Experience in the academic world and with international scientific cooperation. Established profile in the international community and successful track record of fund raising. Knowledge and appreciation of and commitment to the principles and ideals of the United Nations and with a wide grasp of the problems of the modern world. Capability to maintain close cooperation with individuals, governments and with research and teaching institutions worldwide. Great drive and initiative to achieve the goals of UNU. Some knowledge of other United Nations official languages is desirable.

Remuneration: Competitive net salary (tax-exempted) and allowances at a senior level (USG) within the United Nations system, including post adjustment. Post adjustment is subject to change. The post carries the standard set of United Nations entitlements/benefits, including participation in the United Nations Joint Staff Pension Fund, the possibility of participation in a health insurance programme, education grant for eligible children, removal expenses and home leave. For more information please visit: http://sas.undp.org/portal/alias_SAS/lang_en-GB/tabID_3379/DesktopDefault.aspx.

Address applications to: UNU Rectorship Nominating Committee, United Nations University, 53-70, Jingumae 5-chome, Shibuya-ku, Tokyo 150-8925, Japan. Nominations or letters of application, including full curriculum vitae and list of publications, and should clearly indicate the above reference number. Applications must be sent by post to the above address. No email or fax applications will be accepted.

Application deadline: 15 June 2011

It is expected that the appointee will take up the position by September 2012. The initial appointment will be for a five-year term, with the possibility of a second term. Applications from women candidates are particularly encouraged. Please visit www.unu.edu/employment for more information.

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Georgia Health Sciences University

Senior Vice President for Research

Georgia Health Sciences University is conducting a nationwide search for a Senior Vice President for Research. The Search Committee invites letters of nomination, applications (letter of interest, complete CV, and a list of five references), or expressions of interest to be submitted to the search firm assisting Georgia Health Sciences University. Confidential review of materials will begin immediately and will continue until the appointment is made. It is preferred, however, that all materials be submitted by **April 13, 2011**. For a complete position description, refer to Current Opportunities on www.parkersearch.com. Materials should be submitted to:

**Laurie Wilder, Senior Vice President
and Managing Director
Parker Executive Search
lwilder@parkersearch.com
Phone: 770-804-1996 x 102 / Fax: 770-804-1917**

Georgia Health Sciences University is an Equal Employment, Equal Access, and Equal Educational Opportunity and Affirmative Action Institution. It is the policy of the University to recruit, hire, train, promote and educate persons without regard to age, disability, gender, national origin, race, religion, sexual orientation or veteran status.



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Nutritional Sciences UNIVERSITY OF TORONTO

Assistant Professor in Nutritional Sciences

The Department of Nutritional Sciences in the Faculty of Medicine invites applications for a tenure-track Assistant Professorship. The research interests of the Department range from basic science to clinical investigation and population health. Applications are encouraged from candidates with an excellent record of research accomplishments in any one of our four core research platforms: healthy human development and aging; nutrigenomics and personalized nutrition; chronic disease prevention and treatment; and nutrition, food and public policy, profiled on www.utoronto.ca/nutrisci/. Successful candidates will be expected to mount an independent, externally funded research program and to participate in some teaching at the undergraduate or graduate level.

Applicants should send curriculum vitae, description of research interests and the names and addresses of 3 references by **April 30, 2011** to:

**Dr Mary L'Abbé, Chair,
Department of Nutritional Sciences
Faculty of Medicine, University of Toronto
150 College St., FitzGerald Building
Toronto, ON, Canada M5S 3E2
Mary.Labbe@utoronto.ca
Phone: 416-978-7235 Fax: 416-971-2366**

The University of Toronto is strongly committed to diversity within its community and especially welcomes applications from visible minority group members, women, Aboriginal persons, persons with disability, members of sexual minority groups and others who may contribute to further diversification of ideas. All qualified candidates are encouraged to apply; however, Canadians and permanent residents will be given priority.

UNIVERSITY OF LOUISVILLE

BIRTH DEFECTS CENTER

[HTTP://LOUISVILLE.EDU/BIRTHDEFECTSCENTER/](http://LOUISVILLE.EDU/BIRTHDEFECTSCENTER/)

Tenure-Track Faculty Positions Molecular Determinants of Embryonic Development

The University of Louisville Birth Defects Center, a NIH-funded Center for Biomedical Research Excellence, invites applications for a tenure-track faculty appointment at the level of Assistant, Associate or Full Professor. Candidates should have an active research program in the area of molecular or molecular genetic mechanisms of normal and/or abnormal embryonic development, or developmental origins of health and disease. The successful candidate's research program should complement the Center's existing strengths in: epigenetic determinants of normal and abnormal embryonic development; developmental origins of adult disease; and molecular and molecular genetic aspects of craniofacial development. Candidates must have a Ph.D. degree, or the equivalent, in a biomedically related field, at least two years of postdoctoral training, and a sound research program reflected by a strong publication record. Successful candidates will be expected to establish and maintain an independent and innovative research program that attracts extramural funding. Applicants for advanced academic rank are expected to have a strong history of extramural funding and lead a research program that is currently supported by extramural funding.

Applicants must apply online at: www.louisville.edu/jobs for position #26765, at which time a curriculum vitae must be uploaded. In addition, applicants should send a single pdf document containing: (1) a letter of intent, (2) a statement of current research activities and plans, and (3) names and contact information for three references to: **Dr. Bob Greene; Chair, Department of Molecular, Cellular and Craniofacial Biology; Director, Birth Defects Center, e-mail: Dr.Bob.Greene@gmail.com.**

The University of Louisville is an Affirmative Action, Equal Opportunity, Americans with Disabilities Employer, committed to diversity and in that spirit, seeks applications from a broad variety of candidates



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JANELIA CONFERENCES

FALL 2011

The Janelia Farm Research Campus is pleased to announce its tenth season of conferences. These small, intense conferences are intended to foster rapid scientific advances and collaborative interactions. All participants are expected to contribute to the intellectual content of the meetings. The Howard Hughes Medical Institute fully supports the Janelia Conferences—there are no registration, accommodation, or dining fees for participants. The conference organizers invite all participants, selecting some from an open pool of applicants.

High Resolution Circuit Reconstruction ■ September 11–14, 2011
Organizers: Mitya Chklovskii (HHMI/Janelia Farm) and Winfried Denk (Max Planck Institute for Medical Research)

Biolmage Informatics II ■ September 18–21, 2011
Organizers: Gene Myers and Hanchuan Peng (both of HHMI/Janelia Farm), Michael Hawrylycz (Allen Institute for Brain Science), Jean-Christophe Olivo-Marin (Institut Pasteur), and Badri Roysam (University of Houston)

Control of Neuronal Identity ■ October 9–12, 2011
Organizers: Tzumin Lee and Jim Truman (both of HHMI/Janelia Farm), Chris Doe (HHMI/University of Oregon), and Sally Temple (New York Neural Stem Cell Institute)

Single Molecules Meet Systems Biology ■ October 23–26, 2011
Organizers: Taekjip Ha (HHMI/University of Illinois at Urbana-Champaign), Su-A Myong (University of Illinois at Urbana-Champaign), Arjun Raj (University of Pennsylvania), and Sunney Xie (Harvard University)

The Neural Basis of Motor Control
October 30–November 2, 2011
Organizers: Silvia Arber (University of Basel), Adam Hantman (HHMI/Janelia Farm), and Keir Pearson (University of Alberta)

Janelia Farm offers scholarships to graduate students who would otherwise be unable to participate in the conferences. The scholarships fund students who are from groups that are underrepresented in the sciences or who come from disadvantaged backgrounds.

Information:

www.hhmi.org/janelia_conf/sci

Application deadline: May 13, 2011



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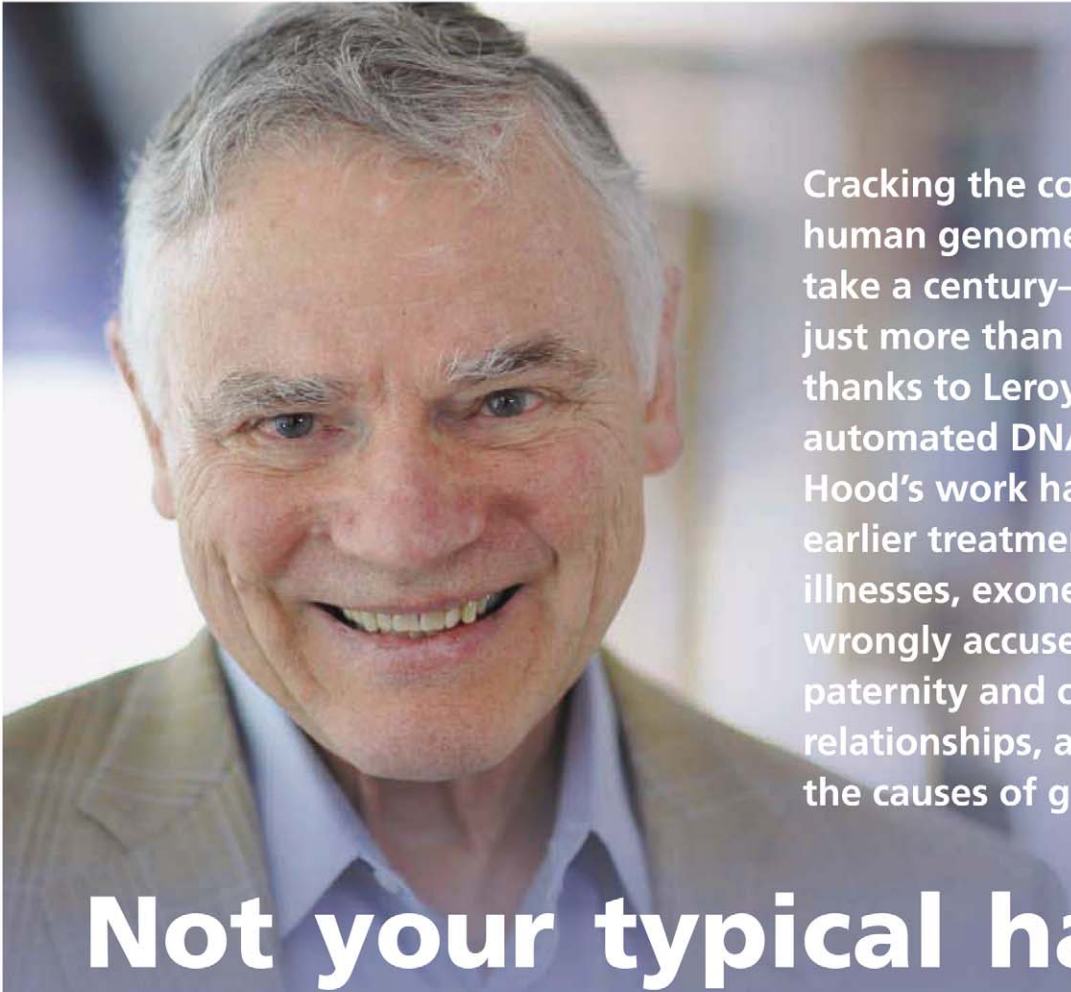
- Do industry and academic careers require different skill sets?
- Do industry jobs have better compensation? Less autonomy?
- Do academic scientists have less work/life balance?

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Produced by the *Science*/AAAS Business Office.

A close-up portrait of Leroy Hood, an older man with grey hair, smiling. He is wearing a light blue collared shirt under a tan jacket.

Cracking the code of the human genome didn't take a century—it took just more than a decade, thanks to Leroy Hood's automated DNA sequencer. Hood's work has enabled earlier treatment of countless illnesses, exonerated the wrongly accused, established paternity and other family relationships, and helped find the causes of genetic diseases.

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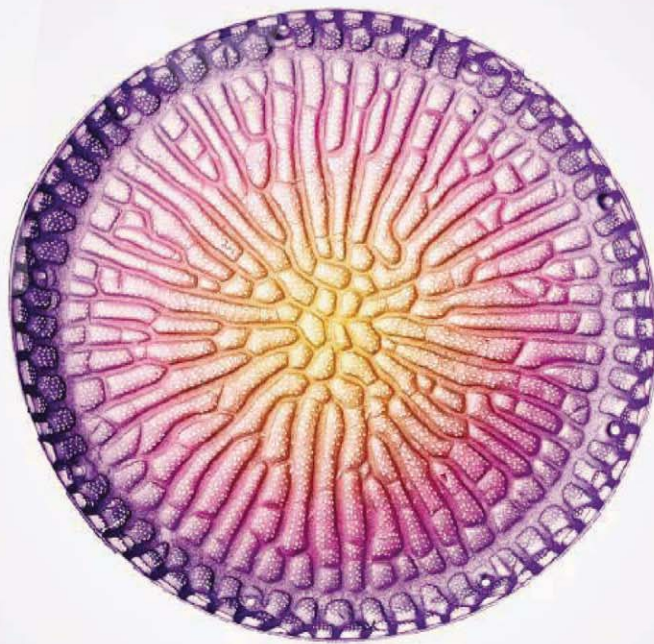
Leroy Hood, M.D., Ph.D.
*President and Co-Founder
Institute for Systems Biology, Seattle
2011 Fritz J. and Dolores H. Russ Prize Recipient*



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